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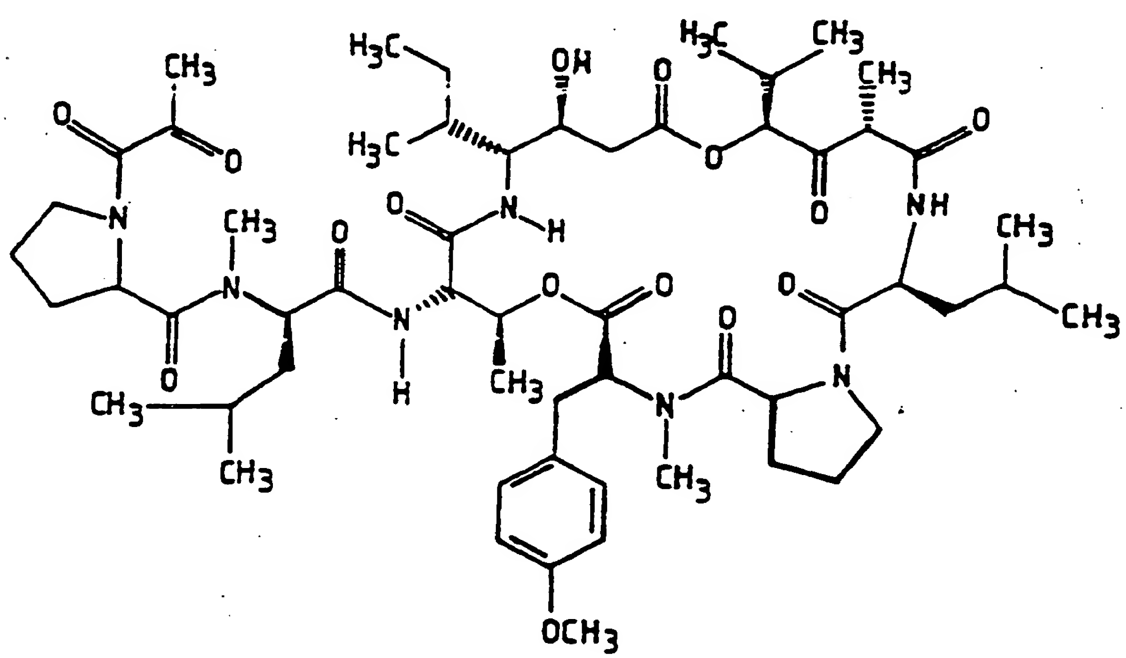

(I)

(57) Abstract

Dehydrodidemnin B with useful biological activity is of formula (I). It can be isolated from natural sources or synthesised, and forms active derivatives.

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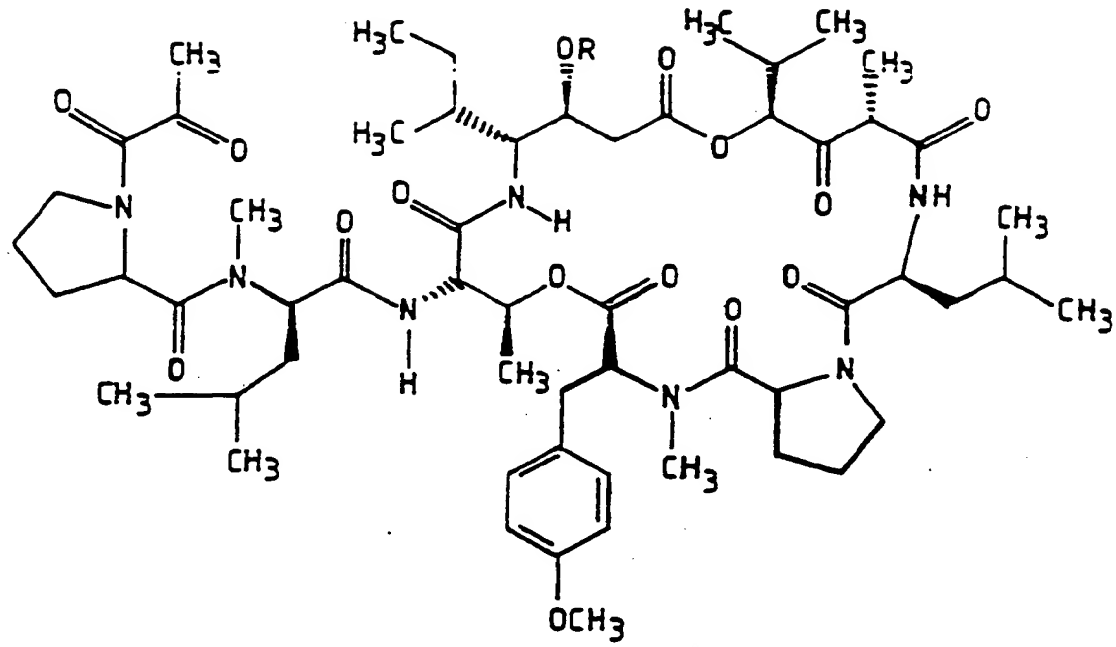
DEHYDRODIDEMNIN B

This invention relates to dehydrodidemnin B and, in particular, to the isolation of dehydrodemnin B, a cyclic depsipeptide, from a tunicate of the Ascidiacea class. This novel compound has been shown to have antiviral, antitumoural and cytotoxic activities.

The didemnins form a class of cyclic depsipeptides which have been isolated from various species of the Trididemnum genus. They have been shown to have potent activity against viruses and tumour cells (Rinehart, Jr. K.L. et al, J. Am. Chem. Soc. (1981), 103, 1857-59). Didemnin B, up to now the most active compound of this class, has been shown to have potent immunosuppressive activity (Montgomery, D.W., Zukoski, C.F., Transplantation (1985), 40, 49-56) and a more potent inhibition of binding of prolactin to human lymphocytes than other didemnin compounds (Montgomery, D.W. et al, Fed. Prac, (1987), 44, 634).

This invention provides a novel and more active compound of this class, namely dehydrodidemnin B (DBB), unexpectedly isolated from the Mediterranean tunicate Alpidium albicans, of the formula where R=H:



and its possible derivatives with the same class of biological activity, where R could be Acyl, Alkyl or Aryl.

This compound is characterised by the following properties, considering also that, in solution, two conformers at least are possible:

TLC R_f = 0,4; 0.35 (Silica gel, 2:3, CH_2Cl_2/ETOAc); 0,5; 0.44 (Silica gel; 9:1, $CHCl_3$/MeOH).

RP-HPLC t_R = 10,7; 11.9 min (Spherisorb C_{18} column, 250 mm x 10 mm, 10µm particle size, 9.1, MeOH/H_2O; 2mL/min).

$[\alpha]D25^{\circ} = -86^{\circ}$ (ε1, MeOH)

HR FABMS (M + H) $C_{57}H_{88}N_7O_{15}$ m/z calcd. 1110.6366); (M-side chain + H): $C_{42}H_{66}N_5O_{11}$ m/z calcd. 816.4781 (found 816.4755); (M - side chain): $C_{15}H_{23}N_2O_4$ m/z calcd. 295.1657 (found 2951657).

IR ($CHCl_3$) ν_{max} cm-1: 3680, 3600. 2970, 2940, 2880, 1740, 1650, 1605, 1540, 1510.

^1H NMR ($CDCl_3$, δ, ppm): 7.82 (d, J=9 Hz, IH); 7.79 (d, J=9 Hz, 1H); 7.62 (d, J=6 Hz, 1H); 7.21 (d, J=9 Hz, 1H); 7.19 (d, J=9 Hz, 1H); 7.08 (d, J=8.5 Hz, 2H); 6.85 (d, J=8.5 Hz, 2H); 3.77 (s, 3H); 3.13 (s, 3H); 3.08 (s, 3H); 2.54 (s, 3H); 2.50 (s, 3H); 2.1 (s, 3H); 2.02 (s, 3H); 0.82-0.88 (overlapped d and t, 3OH).

^{13}C NMR ($CDCl_3$, δ, ppm): 204.93 (s); 204.77 (s); 201.23 (s); 197.55 (s); 173.05 (s); 172.36 (s); 171.84 (s); 171.21 (s); 171.16 (s); 170.59 (s); 169.58 (s); 169.51 (s); 169.35 (s); 168.36 (s); 168.28 (s); 161.31 (s); 161.06 (s); 158.64 (s); 158.62 (s), 130.31 (d); 114.12 (d); 114.01 (d); 81.47 (d), 81.43 (d); 70.68 (d); 70.33 (d); 67.97 (d); 67.76 (d); 66.38 (d); 66.22 (d); 60.39 (t); 50.88 (d); 57.80 (d); 57.45 (d); 57.26 (d); 57.18 (d); 57.12 (d); 55.61 (d); 55.57 (d); 55.26 (q); 54.65 (d); 49.55 (d); 49.49 (d); 48.85 (t); 48.41 (t); 46.98 (t); 41.29 (t); 41.24 (t); 38.78 (q); 38.74 (q); 38.68 (q); 36.42 (t); 36.22 (t); 34.06 (d); 33.99 (d); 33.96 (t); 31.57 (d); 31.38 (q); 31.34 (q); 31.30 (q); 30.69 (d); 29.68 (t); 29.64 (d); 27.98 (t); 27.94 (t); 27.30 (t); 27.17 (t); 27.08 (t); 25.91 (t); 25.87 (t); 25.73 (d); 25.68 (d); 25.63 (d); 25.52 (d); 25.48 (d); 24.80 (q); 24.70 (q); 24.44 (q); 24.31 (q); 22.21 (q); 22.12 (q); 21.92 (q); 21.79 (q); 21.76 (q); 19.46 (q); 17.76 (q); 17.72 (q); 17.18 (q); 16.87 (q); 16.08 (q); 15.62 (q); 15.48 (q); 15.05 (q); 12.55 (q); 12.50 (q).

The structure determination of DDB was accomplished by comparison of mass spectrometry [low and high resolution FABMS (Rinehart, Jr,. K.L. et al. Pure and Appl. Chem. (1982), 54, 2409-2424)] and NMR data with other didemnin data, and confirmed by synthesis of DDB involving coupling of natural didemnin A with the appropriate side chain. The low resolution FAB mass

spectra showed peaks at m/z 1110 (M - H), 816 (M + 2H - side chain) and 295 (side chain). The lack of two mass units in the molecular ion and side chain peaks, in addition to the same m/z ration for the ring, suggested that the difference between dehydrodidemnin B and didemnin B was represented by one more degree of unsaturation in the side chain. The molecular formula deduced from high resolution FABMS was $C_{57}H_{88}N_7O_{15}$ (M + H, 2.8 mmu); and for the fragment ions corresponding to the ring and the side chain; $C_{42}H_{66}N_5O_{11}$ (0.4 mmu) and $C_{15}H_{23}N_2O_4$ (2.6 mmu), respectively. Tandem mass spectrometry on these peaks showed the typical cleavage pattern of didemnins.

From the NMR data, the presence of peptide linkages was indicated by peaks near δ 8 ppm and the methyl signals corresponding to the amino acid residues. Even though some of these peaks are doubled or tripled due to the presence of, at least, two main conformers in solution at room temperature , these peaks are very similar to those of didemnins. The main difference observed between DDB and didemnin B is the methyl singlet peak at 2.04 ppm which could be assigned to a methyl ketone and the absence of the signal corresponding to the α-proton of the hydroxyl group in the lactyl moiety at 4.3 ppm.

The compound of this invention has been shown to inhibit in vitro L1210 and P-388 mouse leukemia cells; L-929, mouse areolar and adispose tissue, B-16, mouse melonoma cells; A-549, human lung carcinoma cells; HeLa, human cervix epitheloid carcinoma cells and KB, human oral epidermoid carcinoma cells, and in vivo. P-338, mouse leukemia cells, Lewis lung carcinoma cells and B-16 melanoma cells. Thus, DDB is useful as an antitumour agent and therefore is useful inhibiting the growth of tumour cells in mammals exhibiting such tumour cells.

The following table summarises the IC_{50} values for each line cells in vitro

Table 1

Line Cells	IC_{50} (ng/mL)
L-1210	0.3
P-388	0.175
L-929	1.9
B-16	0.225
A-549	0.5
HeLa	0.5
KB	5.6

The following table shows the % T/C in vivo after administration of DDB:

Table 2

Dehydrodidemnin B in vivo Activity

Compound	Control	DDB	DDB
Dose (μ/kg/inj)		160	80
Schedule and Route	QD 1-9, IP	QD 1-9, IP	QD 1-9, IP
P-388, Median Survival Time, Days	10.0	21.0	19.5
P-388 % T/C [a]	100	210	195
Levis Lung, Mean Tumour Volume mm^3	1512	0	189
Levis Lung % T/C[b]	1.00	0.00	0.13

B-16 Melanoma, Median Survival Time, Days	17.0	>27.0	>27.0
B-16 Melanoma, % T/C[a]	100	>158	>158

a. Significant activity T/C ≥ 125. b. Significant activity ≥ 0.40

Dehydrodemnin B, like didemnin B, (Montgomery, D. W., Zukoski, C. F., Transplantation (1985), 40, 49-56), is a powerful immunomodulator.

Dehydrodidemnin B has also shown activity against Herpes simplex virus, type 1, in CV-1 cells (monkey kidney cells); thus it is also useful as an antiviral agent. The IC_{50} determined was 60 ng/mL (e.g 10 fold greater than for L-1210 cells) and 1 µg/mL, respectively.

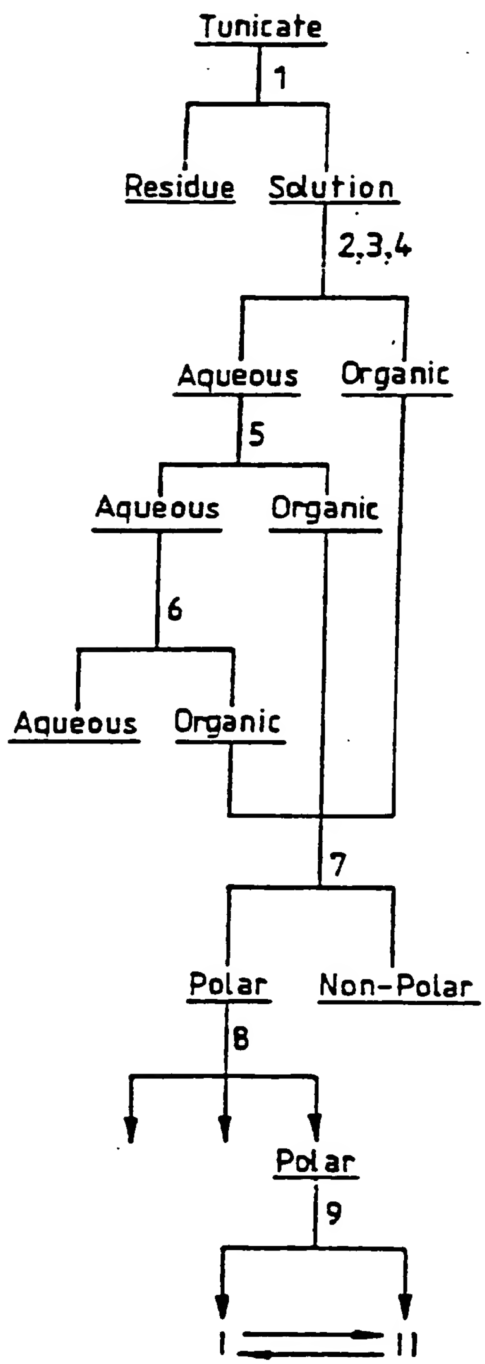
The compound of present invention is preferably presented for administration to humans and animals in unit dosage forms in pharmaceutically appropriate carriers containing the active ingredient in the appropriate quantity.

Illustratively, dosage levels of the administered active ingredient can be intravenous 0.05 to about 50 mg/Kg, intraperitoneal, subcutaneous and intramuscular 1 to 100 mg/Kg; oral 1 to 150 mg/Kg of animal (body) weight.

The administration of DDb is useful to inhibit the growth of cancer cells in animals or humans bearing a neoplastic disease, for example, acute myelocytic leukemia, acute lymphocytic leukemia, malignant melanoma, adenocarcinoma of the lung, small cell carcinoma of the lung, and the like.

Such a compound can be isolated (Isolation Scheme) from tunicates of the Ascidiacea class Subphylum Urochordata as described in the following chart.

Chart 1



1. Polar Organic Solvent.
2. Evaporation.
3. Partially Miscible Organic Solvent Mixtures.
4. Salty Solution (10-40%)
5. Medium-Polar Chlorinated Organic Solvent.
6. Medium-Polar Org. Sol./ Water Liq-Liq Partition.
7. Partially Miscible Organic Solvent Partition.
8. Silica gel Step-gradient Chromatography.
9. Reversed-Phase HPLC

In particular, the compound can be isolated from tunicates of the genus <u>Aplidium</u>, and more especially from the species <u>Aplidium albicans</u>. The species is found in the Iberian Mediterranean Coast as well as in the Balearic Islands. The species has been also found in Great Britain, English Channel as well as in the Africa Coast and Portugal. It seems to prefer detritic, coralligenic and sciafilae algae communities. They also can be found in more photophylic habitats.

Colonies of the tunicate are generally flat and lobed (2.5 cm diameter). It is jelly like, totally encrusted with sand which confers a sandy colour to the colony. Zooides are of a whitish colour 10 mm long; the oral siphon has 6 lobes, and the cloacal languet is trifid, which is a species characteristic. Generally there are 10-11 rows of stigmas. The stomach has 6 marked folds. Gonads are of the family type with one or several ovocites below the digestive track and numerous testicular follicles forming one or double row in the post abdomen. Larvea are incubated in the number of 1 to 9 in the artrial cavity; they have 3 cupping-glasses and several vesicular formations in the anterior part.

Thus in a typical procedure in accordance with the present invention, isolation method generally comprises alcoholic extraction of the homogenized tunicate and selective purification of the desired DDB.

In the illustrative scheme shown in Chart 1, the tunicate was extracted with MeOH, filtered and dissolved in MeOH: Toluene 3:1 and partitioned with 10% NaNo3. The aqueous layer was successively extracted with CH_2Cl_2, EtOAc and n-BuOH. The organic fractions were combined after monitoring by normal-phase TLC developed $CHCl_3$: MeOH 9:1, affording a 2:1 (v/v) and

the activity was concentrated in the methanolic layer. This polar fraction is passed through Silica gel Step-gradient Chromatography. The last fraction is further purified by reverse phase HPLC at a flow rate of 2ml/min. Two mean peaks were collected and readily interconverted to a mixture of I and II, to establish an approximately 1:1 ratio.

The DDB can also be prepared by total synthesis, or hemisynthesis from natural Didemnin A, following in both cases standard procedures of protection and activation in peptide chemistry.

Pyruvic acid + L-Pro	gives	Side Chain
Side Chain + Didemnin A	gives	Dihydrodidemnin B

Thus for example, Pro-OBzl, in DMF is mixed with pyruvic acid and HOBt, and DCC in CH_2Cl_2 added. The reaction product can be purified and shows the chemical and physical properties corresponding to Pyruvyl-Pro-OBzl.

To a solution of this last product in CH_2Cl_2, EDC and then Didemnin A was added. The evaporated residue is purified yielding DDB having chemical, physical, spectroscopical and biological characteristics in accord with natural Dehydrodidemnin B.

Apart from DDB itself, the present invention extends to derivatives of DDB, comprising acylated, alkylated or arylated derivatives of DDB, where R could be a group COR′ or R′, where R′ represents the following sustituents:

CH_3,, CH_2R_1, CH_2R_1, $CHR_1R_2R_3$ or
C_6H_5-
where R_1, R_2, R_3, could be alkyl (either linear or branched), aryl or alkylaryl, the aryl groups, bearing

or not the substituents described under R'. The residues R_1, R_2, R_3 could be either the same or different.

In general, such derivatives from DDB of this kind, are expected to show similar biological activity to that of DDB itself, including specifically antitumoural, antiviral, cytotoxic and immunosuppressive activity.

The derivatives can be more preferably alkyl, aryl or acyl-derivatives, where R' is an aliphatic or aromatic group, more preferably a 1-6 carbon atoms residue.

The acyl derivatives can be obtained by treatment of the parent compound with the corresponding carboxylic anhydride in the presence of pyridine or other nitrogenated organic base; by reaction of DDB with the respective acylchloride; or by dehydration with DCC from DDB and the corresponding carboxylic acid.

In the case of the alkyl or aryl derivatives, R=R', they can be obtained by reaction of DDB with the corresponding halide, in the presence of an alkaline-organic weak base or by dehydration between DDB and alkyl or aryl hydroxiderivative by an organic dehydrating agent.

Instrumentation, Material and Methods

NMR spectra were obtained with a General Electric QE-300 (300 MHz, ^{1}J), a Nicolet NT-360 (360 MHz, ^{1}H) or a General Electric GN 500 (500 MHz, ^{1}H) at the University of Illinois or a Varian Unity 300 (300 MHz, ^{1}H and 75 MHz, 13_C) at Pharma Mar, S.A. (Madrid, Spain) Chemical shifts are reported in ppm referenced to the chloroform peak at δ 7.26 ppm for ^{1}H. FABMS

were measured on a VG Analytical ZAB at the Mass Spectrometry Laboratory of the University of Illinois. GC analyses were carried out using a Varian GC (Model 3700) equipped with an Alltech Associates, Inc., Chirasil-Val II capillary column (25 m x 0.32 mm) with Helium gas carrier at a flow rate of 1.2 ml/min with programmed oven temperature (90°C, 4°C/min, 180°C). Reversed-phase HPLC was performed on a system equipped with an Altex pump (Model 110 A) and a Waters Associates differential refractometer (Model R-401) and an Alltech Spherisorb C18 column (25 cm x 1 cm, particle size 10 µm) with MeOH: H_2O 9:1 as the solvent system.

The following examples illustrate the invention.

Example 1

1. Structure determination

The structure of DDB has been determined by physical and spectroscopic methods.

1.1 Spectroscopic data

TLC R_f = 0.4; 0.35 (Silica gel, 2:3, CH_2Cl_2/EtOAc); 0,5; 0.44 (Silica gel; 9:1, $CHCl_3$/MeOH).

RP-HPLC t_R = 10,7; 11.9 min (Spherisorb C_{18} column, 250 mm x 10 mm, 10 µm particle size, 9:1, MeOH/H_2O; 2mL/min).

[α]D25 = - 86° (c1, MeOH)

HR FABMS (M + H) $C_{57}H_{88}N_7O_{15}$ m/z calcd. 1110.6382 (found 1110.6366); (M-side chain + H): $C_{42}H_{66}N_5O_{11}$ m/z calcd. 816.4781 (found 816.4755): (M - side chain): $C_{15}H_{23}N_2O_4$ m/z calcd. 295.1657 (found 295.1657).

IR ($CHCl_3$) ν_{max} cm^{-1}: 3680, 3600, 2970, 2940, 2880, 1740, 1650, 1605, 1540, 1510.

^{1}H NMR ($CDCl_3$, δ, ppm): 7.82 (d, J=9 Hz, 1H); 7.79 (d, J=9 Hz, 1H); 7.62 (d, J=6 Hz, 1H); 7.21 (d, J=9 Hz, 1H); 7.19 (d, J=9 Hz, 1H); 7.08 (d, J=8.5 Hz, 2H); 6.85 (d, J=8.5 Hz, 2H); 3.77 (s, 3H); 3.13 (s, 3H); 3.08 (s, 3H); 2.54 (s, 3H); 2.52 (s, 3H); 2.50 (s, 3H); 2.1 (s, 3H); 2.02 (s, 3H); 0.82-0.88 (overlapped d and t, 30H).

^{13}C NMR ($CDCl_3$, δ, ppm): 204.93 (s); 204.77 (s); 201.23(s); 197.55 (s); 173.05 (s); 172.36 (s); 171.16 (s); 170.59 (s); 169.58 (s); 169.35 (s); 168.36 (s); 168.28 (s); 161.31 (s); 161.06 (s); 158.64 (s); 158.62 (s); 130.31 (d); 114.12 (d); 114.10 (d); 81.47 (d); 81.43 (d); 70.68 (d); 70.33 (d); 67.97 (d); 67.76 (d); 66.38 (d); 66.22 (d); 60.39 (t); 50.88 (d); 57.80 (d); 57.45 (d); 57.26 (d); 57.18 (d); 57.12 (d); 55.61 (d); 55.57 (d); 55.26 (q); 54.65 (d); 49.55 (d); 49.49 (d); 48.85 (t); 48.41 (t); 46.98 (t); 41.29 (t); 41.24 (t) 38.78 (q); 38.74 (q); 38.68 (q); 36.42 (t); 36.22 (t); 34.06 (d); 33.99 (t); 31.57 (d); 31.38 (q); 31.34 (q); 31.30 (q); 30.69 (d); 29.68 (t); 29.64 (d); 27.98 (t); 27.94 (t); 27.30 (t); 27.17 (t); 27.08 (t); 25.91 (t); 25.87 (t); 25.87 (t); 25.73 (d); 25.68 (d); 25.63 (d); 25.52 (d); 25.48 (d); 24.80 (q); 24.70 (q); 24.44 (q); 24.44 (q); 24.31 (q); 22.21 (q); 22.12 (q); 21.92 (q); 21.79 (q); 21.76 (q); 19.46 (q); 17.76 (q); 17.72 (q); 17.18 (q); 16.87 (q); 16.08 (q); 15.62 (q); 15.48 (q); 15.05 (q); 12.55 (q); 12.50 (q).

1.2 Acetylation of DDB

The structure of dehydrodidemnin B can be confirmed also by comparison of the acetylation product with the acetyl derivative of didemnin B.

Acetylation of DDB with acetic anhydride and pyridine gave a monoacetylderivative.

Low resolution mass spectrum showed peaks at ml z 1153.5 (M + H), 859.0 (M + 2H - side chain) and 295.4 (side chain), indicating the loss of one of the two possible sites of acetylation with respect to didemnin B, and that the missing site is the hydroxyl group of the lactyl moiety in the side chain.

1.3 N-Trifluoroacetyl methyl esters of amino acid Residues

The structure of DDB can also be determined by identification of the individual subunits by total hydrolysis and conversion of the amino acids to their N-trifluoroacetyl methyl esters and analysis by GC.

The amino acids were identified by their retention times and comparison of authentic samples obtained from the conversion of didemnin B to the N-trifluoroacetyl methyl esters of the amino acids.

t_R (min): L-Threonine (1.23); D-N-Me-Leucine (1.70); L-Leucine (2.05); L-Proline (2.38); (3S,4R,5S)-Isostatine (3.15, 4.13, 4.77); L-N,O-Me_2-Tyrosine (6.75).

A mixture of DDB and glass-distilled HCl was heated during 18 hours at 110°C in a sealed Teflon-lined screw-capped .ial. The solvent was removed under a steam of N_2 gas.

The hydrolysate was treated with MeOH/Acetyl chloride during 1 hour at 110°C. The solution was cooled to room temperature, the solvent was removed under a stream of N_2 gas. The solid was treated with

a mixture of TFAA/TFA during 15 min at 110°C. The solution was cooled and the solvent evaporated. The residue was dissolved in 2-propanol for GC analysis

Example 2

Biological Activity Assays

2.1 Assay against L-1210 cells (Ascetic fluid from DBA/2 mouse)

L-121- cells were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 mL aliquots of MEN 10C containing the indicated concentrations of drug. All determination were carried out in triplicate. Cells were counted after three drugs was counted daily to ensure that the cells remained in exponential growth over the period of observation.

Growth Inhibition of L-1210 cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	2.9×10^5	0
0.05	2.7×10^5	7
0.1	2.7×10^5	7
0.2	2.1×10^5	28
0.5	1.0×10^5	66
1	2.5×10^4	91
2	6.3×10^3	98

2.2 Assay against P-388 cells (lymphoid neoplasma from DBA/2 mouse)

P-388 cells were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 mL aliquots of MEM 10C containing the indicated concentrations of drug. All determination were carried out in triplicate. Cells were counted after three days of incubation. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period of observation.

Growth Inhibition of P-388 cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	5.63×10^5	0
0.12	3.97×10^5	29
0.25	1.26×10^5	77
0.5	4.47×10^4	92

2.3 Assay against L-929 cells (mouse areolar and adipose tissue).

L-929 cells were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 mL aliquots of MEM 10C. The following day, medium was replaced with 0.5 mL aliquots of MEM 10C. The following day, medium was replaced with 0.5 mL aliquots of MEM 10C containing the indicated concentrations of drug. All determination were carried out in triplicate. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period of observation. Cells were trypsinised and counted 4 days after seeding.

Growth Inhibition of P-929 cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	3.17×10^5	0
1	2.31×10^5	27
2.5	1.13×10^5	64
5	5×10^4	84
10	3.45×10^4	89

2.4 Assay against B-16 cells (mouse melanoma).

B-16 cells were seeded into 16mm wells at 1×10^4 cells per well in 1 mL aliquots of MEM 10C containing the indicated concentrations of drug. All determination were carried out in triplicate. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period for observation. Cells were trypsinised and counted 4 days after seeding.

Growth Inhibition of B-16 cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	1.71×10^5	0
0.16	1.71×10^5	0
0.12	1.27×10^5	25
0.25	8.25×10^4	52
0.5	4.50×10^4	74
1.0	2.88×10^4	83

2.5 Assay against A-549 cells (human lung carcinoma).

A-549 cells were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 mL aliquots of MEM 10C. The following day, medium was replaced with 0.5 mL aliquots of MEM 10C containing the indicted concentrations of drug. All determination were carried out in triplicate. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period of observation. Cells were trypsinised and counted 4 days after seeding.

Growth Inhibition of A-549 cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	8.16×10^4	0
0.25	4.80×10^4	41
0.50	4.00×10^4	50
1.0	2.60×10^4	68
2.5	1.30×10^4	84

2.6 Assay against HeLa cells (human cervix epitheloid carcinoma).

HeLa cells were seeded into 16 mm wells at 1 x 10^4 cells per well in 1 mL aliquots of MEM 10C. The following day, medium was replaced with 0.5 mL aliquots of MEM 10C containing the indicted concentrations of drug. All determination were carried out in triplicate. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period of observation. Cells were trypsinised and counted 4 days after seeding.

Growth Inhibition of HeLa cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	6.25×10^4	0
0.25	5.46×10^4	12
0.50	3.01×10^4	52
1.0	1.90×10^4	70

2.7 Assay against KB cells (human oral epidermoid carcinoma).

KB cells were seeded into 16 mm wells at 1×10^4 cells per well in 1 Ml aliquots of MEM 10C. The following day, medium was replaced with 0.5 ML aliquots of MEM 10C containing the indicted concentrations of drug. All determination were carried out in triplicate. A separate set of cultures without drug was counted daily to ensure that the cells remained in exponential growth over the period of observation. Cells were trypsinised and counted 4 days after seeding.

Growth Inhibition of KB cells by DDB

ng/mL DDB	net increase in cell number	% Inhibition
0	4.50×10^4	0
2.5	4.57×10^4	0
5	2.40×10^4	46
10	1.02×10^4	77

2.8 Assay against HSV-1 (Herpes simplex virus type-1)

16 mm diameter wells were seeded each with 2 x 10^5 CV-1 cells in 1 mL aliquots of MEM 10C. Four days after, cells were infectd with HSV-1 at 10C PFU per well. After adsorption for 1.5 hours, the inoculum was replaced in pairs of wells with 0.5 mL aliquots of MEM 5C containing the indicated concentrations of drug. Cells from two wells without drug were scraped into the medium and frozen 4 hours after infection to provide a baseline for calculating net virus production. The average of these samples was 2.5 x 10^5+ 1.2 x 10^6 PFU per mL. The remaining samples were collected 24 hours after infection.

Inhibition of HSV-1 replication by DDB

ng/mL DDB	net virus produced (PFU/mL)	% Inhibition
0	4.5 x 10^8	0
0.03	3.8 x 10^8	16
0.1	1.5 x 10^8	67
0.3	1.9 x 10^8	96
1	0	100

2.9 Inmunosuppressive Activity

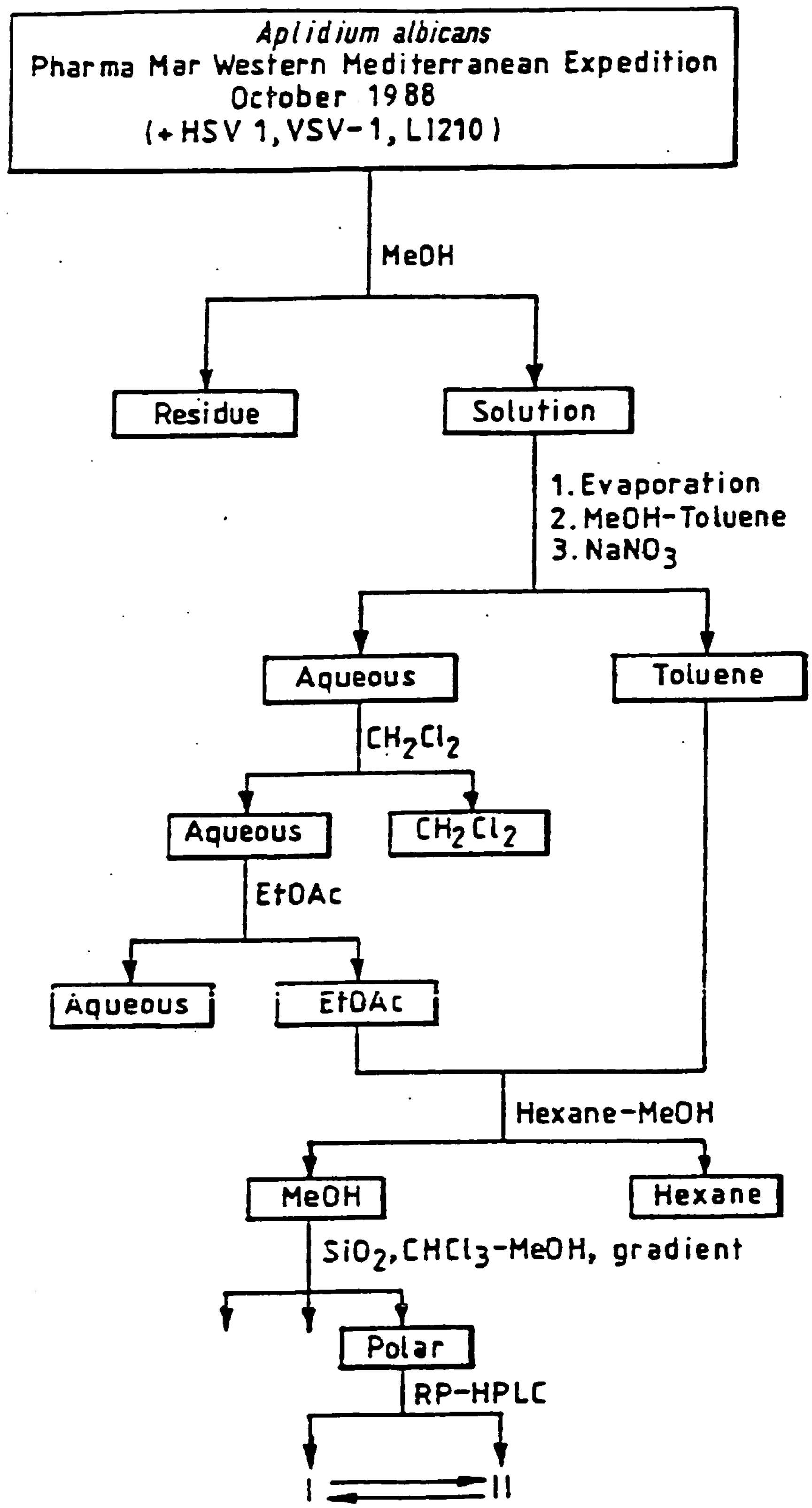
Dehydrodidemnin B is active as an inmunosuppressive agent. In the mixed lymphocyte reaction it suppresses the inmune reaction of murine cells. It also inhibits the growth of murine T-cells and B-cells.

Example 3

Extraction and Isolation

A white solitary tunicate was collected near Ibiza in the Balearic Islands (Spain) and was identified by Dr Xaver Turon of the Universitat de Barcelona, Barcelona (Spain) as Aplidium albicans. A sample is preserved at Centre d' Etudes Avancats, Blanes (Gerona, Spain). Preliminary tests on shipboard inidcated antiviral activity against VSV-1 (Vesicular stomatitis virus). Further studies in the laboratory confirmed the antiviral activity against Herpes simplex virus, type 1 (HSV-1) in monkey kidney cells (CV-1) and also showed cytotoxicity against mouse lymphoid leukemia in vitro (L1210 line cells).

The frozen tunicate was extracted with methanol. Solvent partitioning of the residue afforded three active fractions which were combined according to their similarity in TLC (Thin Layer Chromatography). The crude active fraction was partioned and the activity concentrated in the methanolic layer. The methanol layer was chromatographed by silica gel gravity column (chloroform and chloroform-methanol mixtures), affording one active fraction which was further purified by Reversed-Phase High-Performance Liquid Chromatography ($RPC_{18}HPLC$), affording two peaks (I and II). Analysis by TLC revealed two identical spots in each HPLC fraction. Re-injection of each individual fraction led to two peaks with the same retention times as I and II. Co-injection of I and II confirmed the presence of two identical peaks (possible conformers) in each fraction suggesting a rapid interconversion of I to II and viceversa.


Aplidium albicans
Pharma Mar Western Mediterranean Expedition
October 1988
(+ HSV 1, VSV-1, L1210)
MeOH
Residue
Solution
1. Evaporation
2. MeOH-Toluene
3. $NaNO_3$
Aqueous
Toluene
CH_2Cl_2
Aqueous
CH_2Cl_2
EtOAc
Aqueous
EtOAc
Hexane-MeOH
MeOH
Hexane
SiO_2, $CHCl_3$-MeOH, gradient
Polar
RP-HPLC
I
II

Example 4

Hemisynthesis of DDB from Didemnin A

Dehydrodidemnin B can also be obtained and it structure cofirmed by comparison with a semisynthetic sample prepared byu coupling of the appropriate side chain to natural didemnin A. The data obtained for the semisynthetic sample totally agreed with data for natural DDB.

4.1 Synthesis of Pyruvyl-Pro-OBzl

The hydrochloride salt of Pro-OBzl (10.2 g, 42 mmol) was dissolved in dry DMF (30 ml), neutralized with NMM (N-methylmorpholine, 4.7 mL, 42 mmol) at 0°C, and the solution was mixed with pyruvic acid (8.8 g, 100 mmol) and HOBt (1-hydroxybenzotriazole, 16.8 g, 110 mmol) in CH_2Cl_2 -DMF (80 mL, 8:1). DCC (dicyclohexylcarbodiimide, 22.6 g, 110 mmol) in CH_2Cl_2 (35 mL) was added to the above mixture at 0°C with stirring. The reaction mixture was stirred for 2 hours at 0°C and left overnight at room temperature. DCCl was filtered off and washed with CH_2Cl_2 (20 mL). The filtrate was evaporated to dryness, the residue taken up in EtOAc and washed successively with 5% citric acid, water, 5% $NaHCO_3$ and finally with water to neutral pH. The organic layer was dried (Na_2SO_4) and concentrated. The residue was chromatographed on SiO_2 with hexane-EtOAc (2:1) to give the title compound (11g, 95%).

$[\alpha]_D^{25}$=-78.57 (c 0.14, $CHCl_3$)
R_f=0.63 (19:1, $CHCl_3$/MeOH)
Anal. Calcd. for $C_{15}H_{18}NO_4$ (M + H): 276.1235
Found: 276.1235 (M + H, HRFABMS)

4.2 Synthesis of Pyruvyl-Proline

The protected dipeptide from the previous synthesis (11.0 g, 40 mmol) was dissolved in EtOAc (75 mL) and stirred under hydrogen over Pd/C for 2 h. The catalyst was then filtered off and the filtrate was evaporated to dryness. The residue was crystallized from EtoAc-hexane to give the unprotected peptide (6.9 g, 93%):

$[\alpha]_D^{25}$=-103.99 (c 0.12, $CHCl_3$)
R_f=0.41 (19:1:0.5, $CHCl_3$/MeOH/AcOH
Anal. Calcd. for $C_8H_{12}NO_4$ (M + H): 186/0766
Found: 186.0765 (M + H, HRFABMS)

4.3 Synthesis of Dehydrodidemnin B

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 4.27 g, 22.3 mmol) was added to a solution of Pyruv-Pro (8.2 g, 44.5 mmol) in dry CH_2Cl_2 (40 mL) at 10°C with stirring. The mixture was stirred for 2 h at 10°C and then cooled to 0°C. didemnin A (1.4 g, 1.48 mmol) in CH_2Cl_2 - DMF (10 mL, 4:1) was added, and the clear solution was stirred at 0°C for 2 h and then left in the refrigerator overnight.

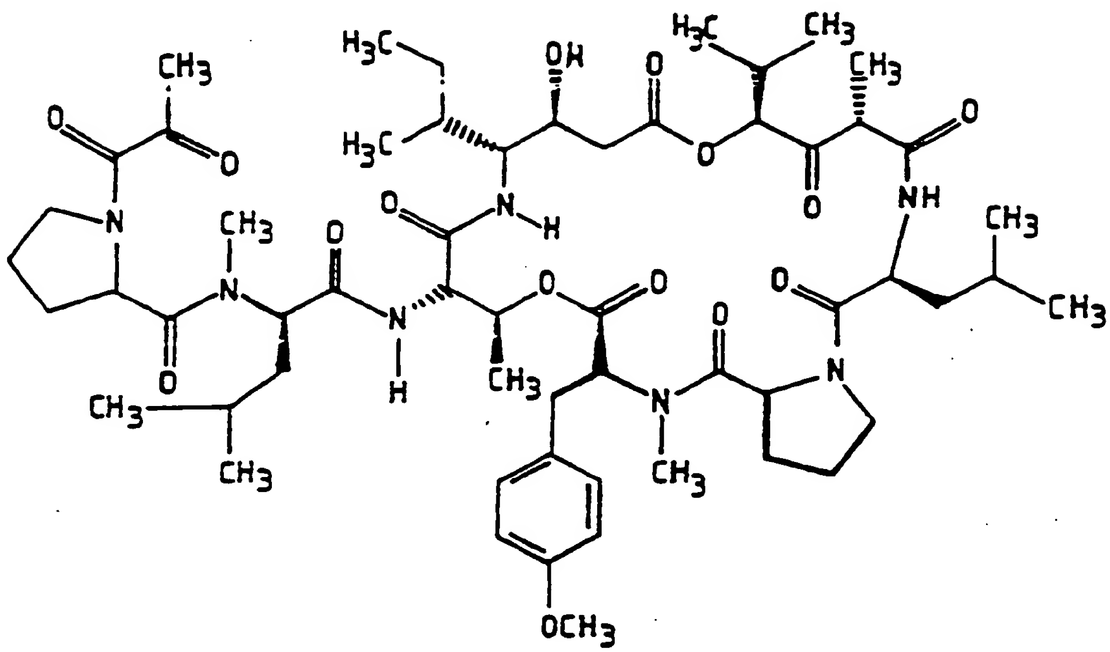
DMAP (4-(dimethylamino)pyridine, 25 mg) was added to the reaction mixture, and it was again left in the refrigerator for 48 h. The solvent was evaporated to dryness, and the residue was taken up in EtOAc and washed with 5% $NaHCO_3$ and water to neutral pH. The organic layer was dried (Na_2SO_4) and concentrated. The residue so obtained was chromatographed on silica gel using $CHCl_3$-MeOH (19:1) to give dehydrodidemnin B (1.4 g, 84%, 2 spots on TLC):

$[\alpha]_D^{25}$=-95.384 (c 0.06, MeOH)
R_f=0.51 and 0.44 (19:1, $CHCl_3$/MeOH)
Anal. Calcd. for $C_{57}H_{88}N_7O_{15}$ (M + H): 1110.6338
Found: 1110.6355 (M + H, HRFABMS)

The same series of reaction can be caried out with slight modifications, in particular EDC can be replaced by DDC with slightly lower yield.

CLAIMS

1. Dehydrodidemnin B of the formula:

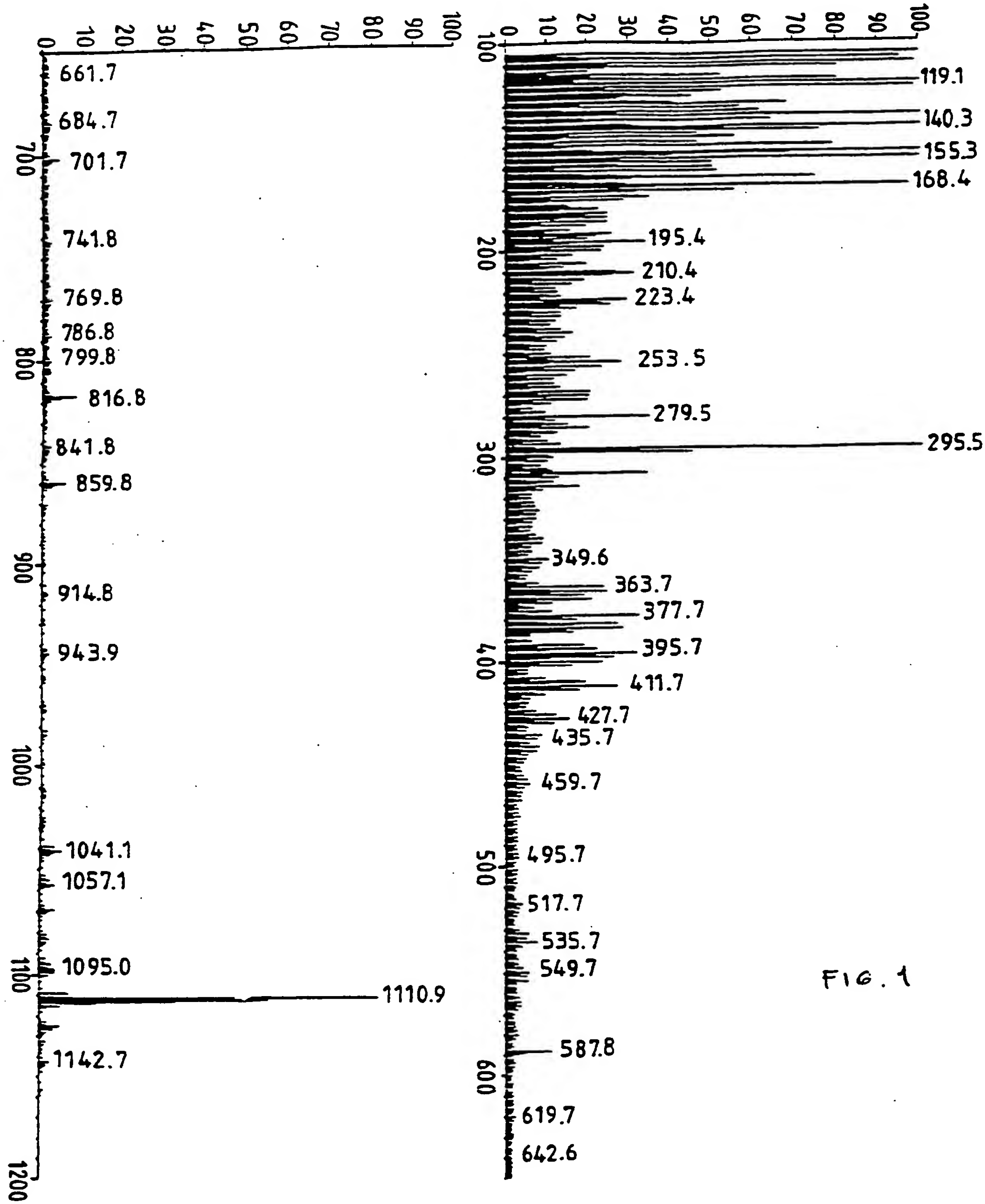


2. A dehydrodidemnin B derivative which is an acyl, alkyl or aryl derivative of dehydrodidemnin B.

3. A pharmaceutical composition which comprises dehydrodidemnin B or an active derivative thereof, together with a pharmaceutically acceptable carrier.

4. A process for preparation of dehydrodidemnin B, which comprises coupling of pyruvyl-proline to didemnin A.

5. A method for isolation of dehydrodidemnin B which comprises extraction from a dehydrodidemnin B-containing tunicate.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799.8
816.8
841.8
859.8
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943.9
1041.1
1057.1
1095.0
1110.9
1142.7
119.1
140.3
155.3
168.4
195.4
210.4
223.4
253.5
279.5
295.5
349.6
363.7
377.7
395.7
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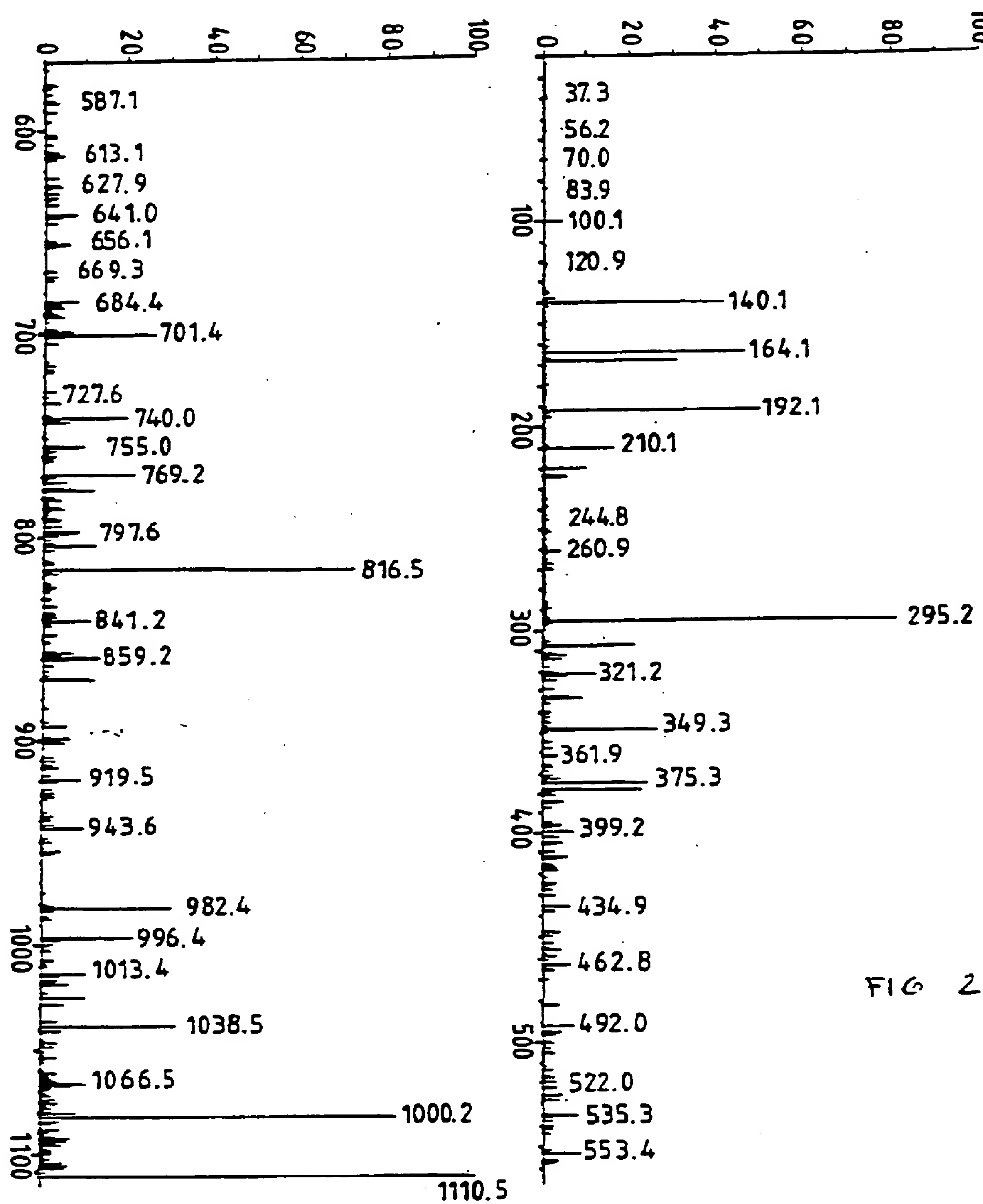
587.8
619.7
642.6

FIG. 1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20
40
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700
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1100
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669.3
684.4
701.4
727.6
740.0
755.0
769.2
797.6
816.5
841.2
859.2
919.5
943.6
982.4
996.4
1013.4
1038.5
1066.5
1000.2
1110.5
100
200
300
400
500
37.3
56.2
70.0
83.9
100.1
120.9
140.1
164.1
192.1
210.1
244.8
260.9
295.2
321.2
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375.3
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492.0
522.0
535.3
553.4

FIG 2

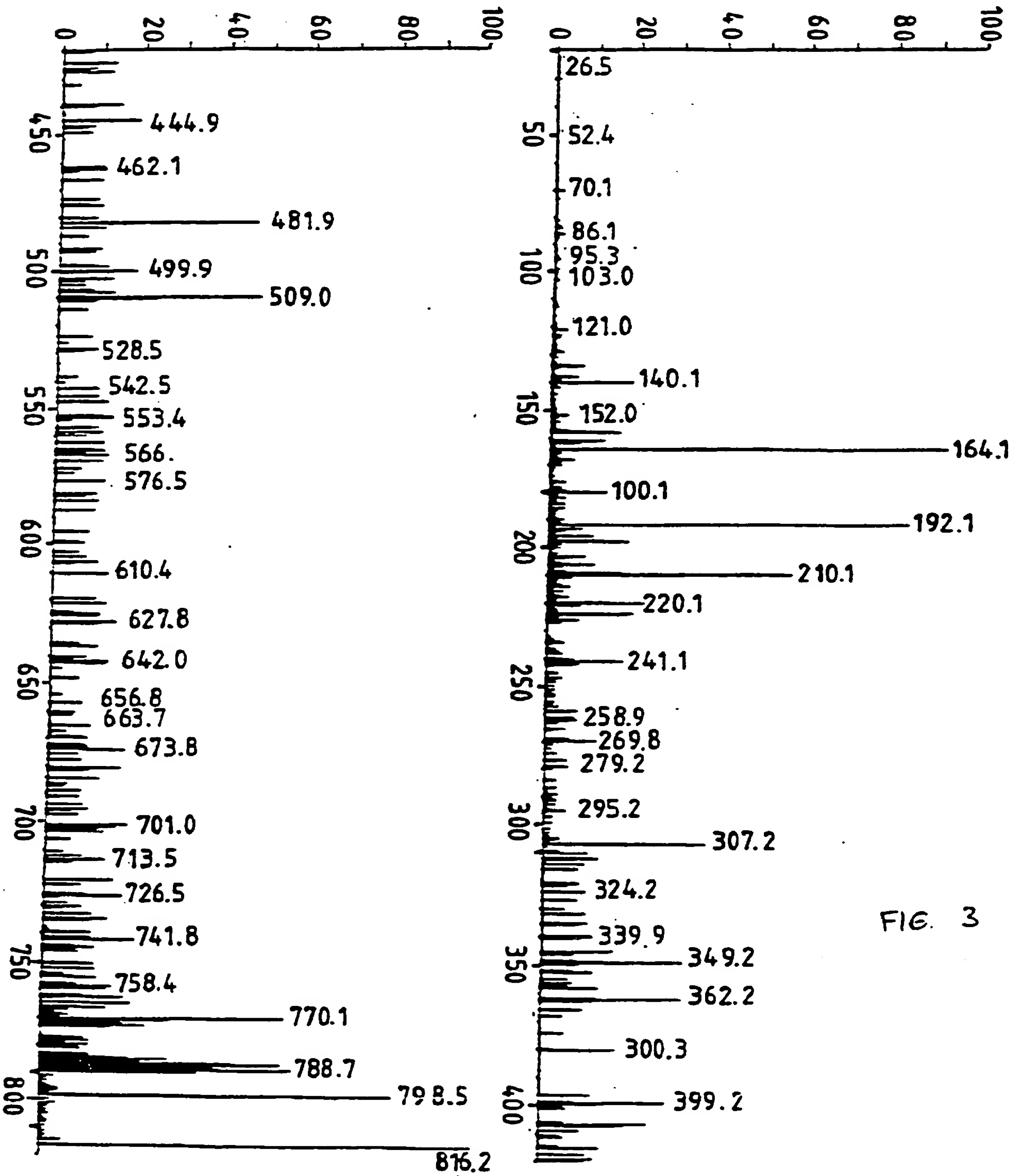

FIG. 3

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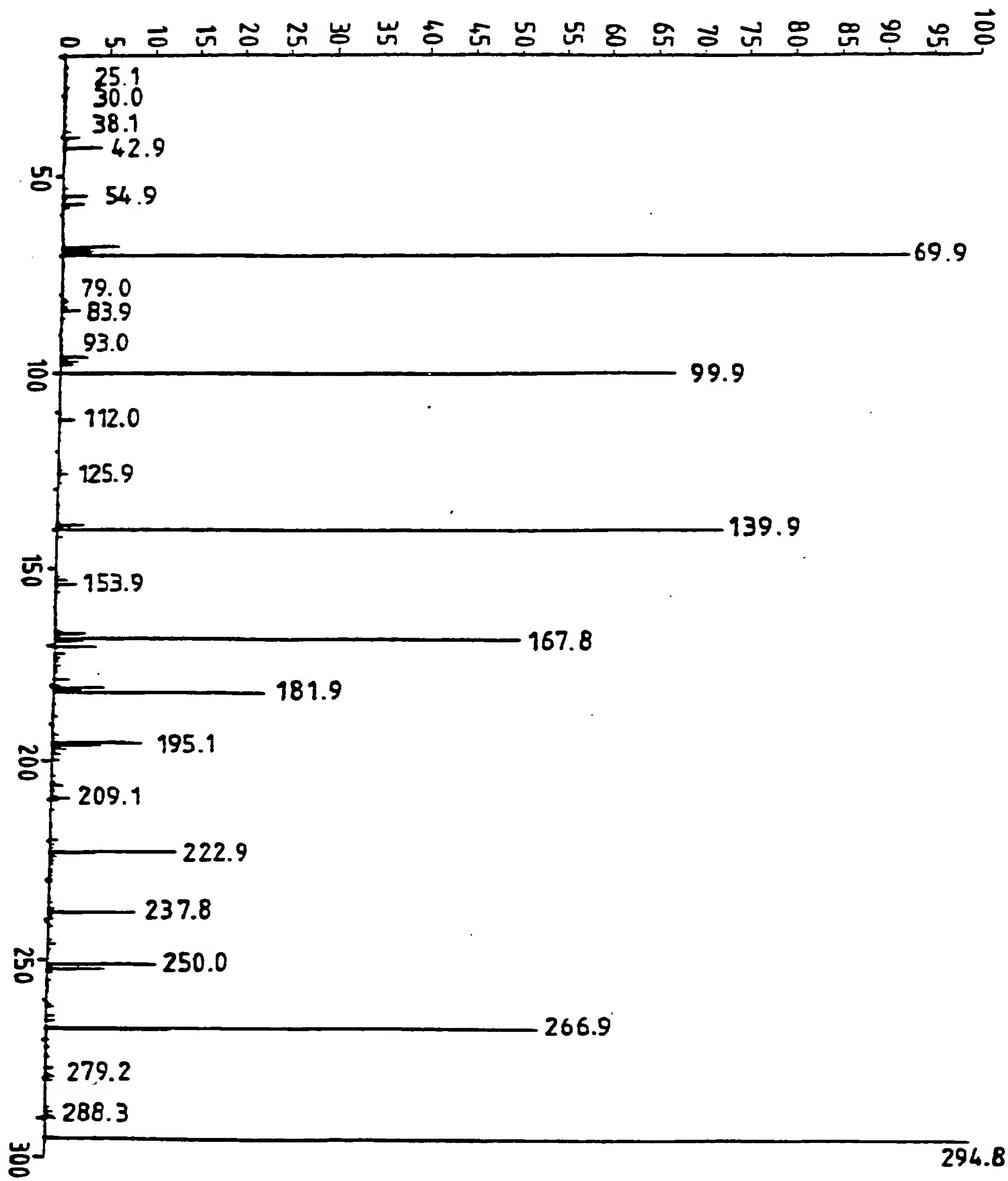

FIG. 4

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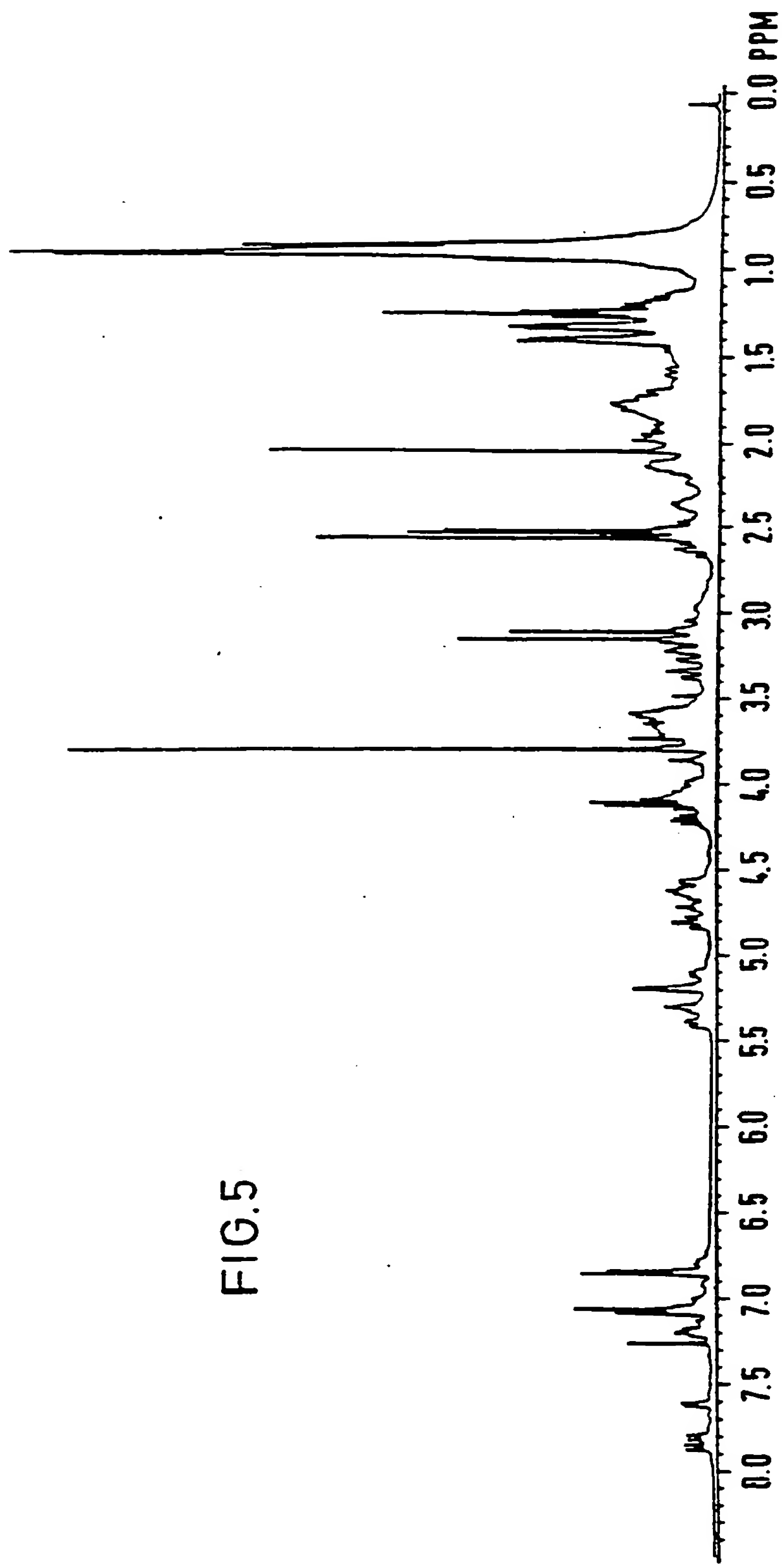

FIG.5

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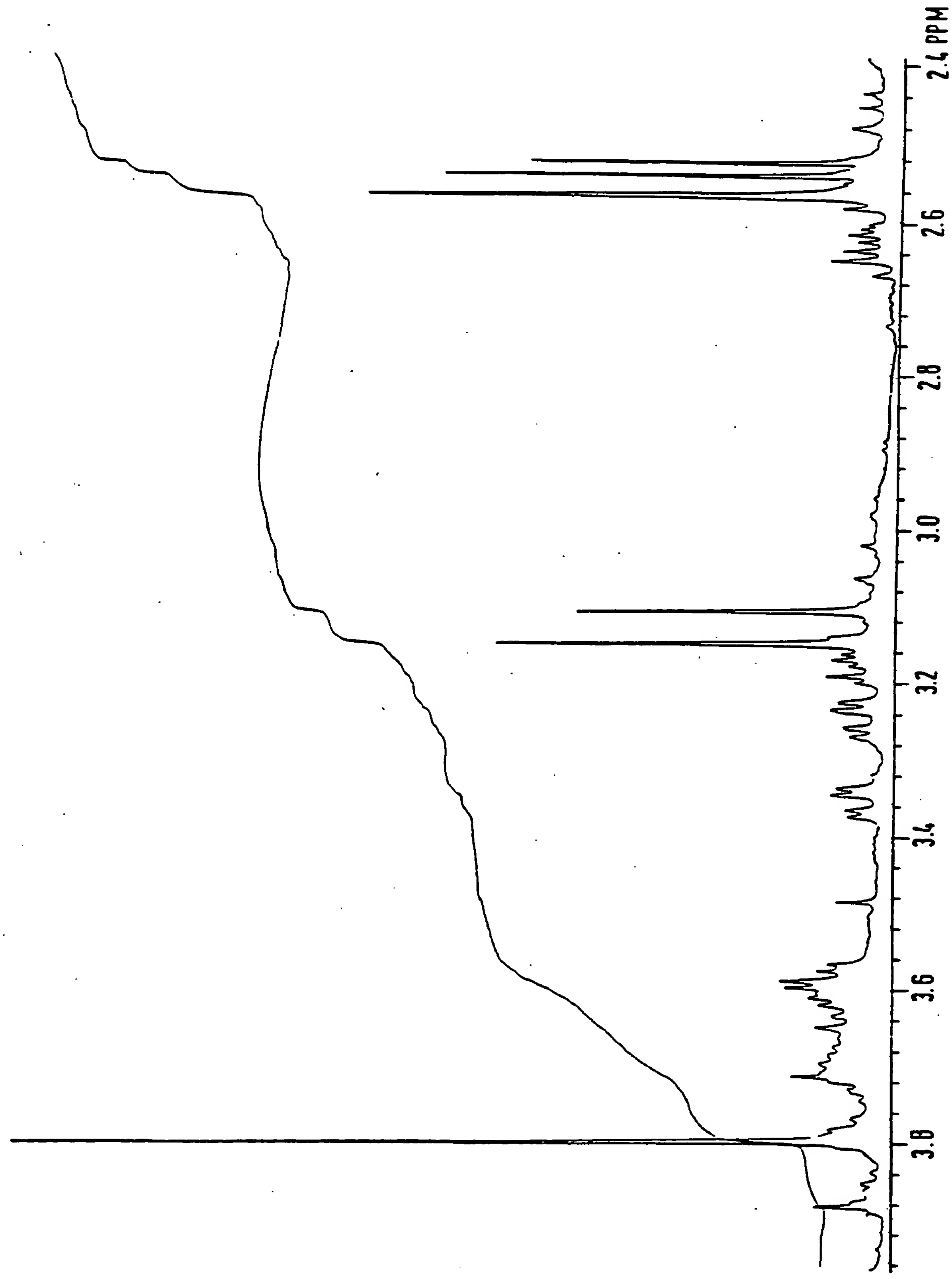

FIG.6

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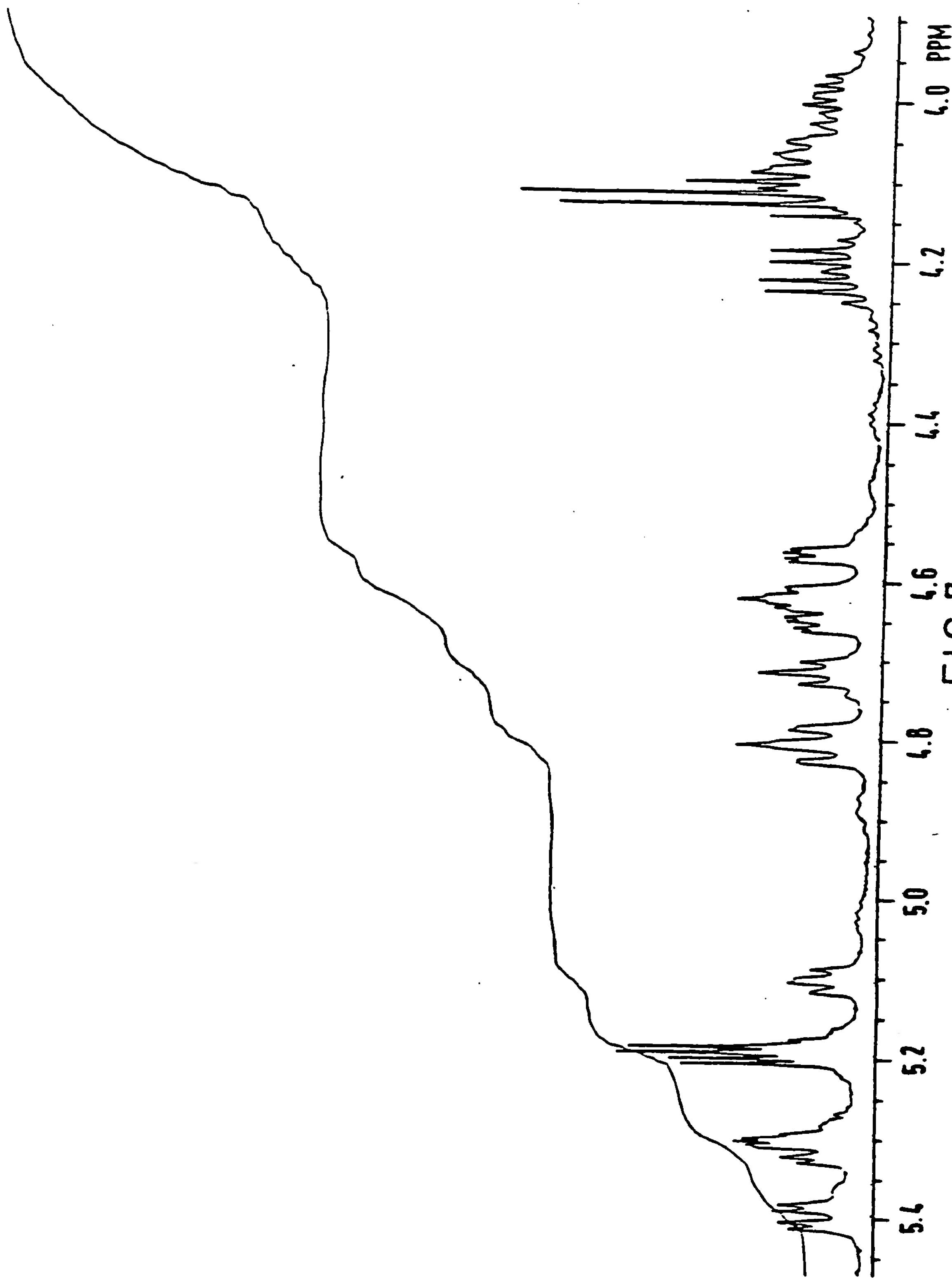

FIG.7

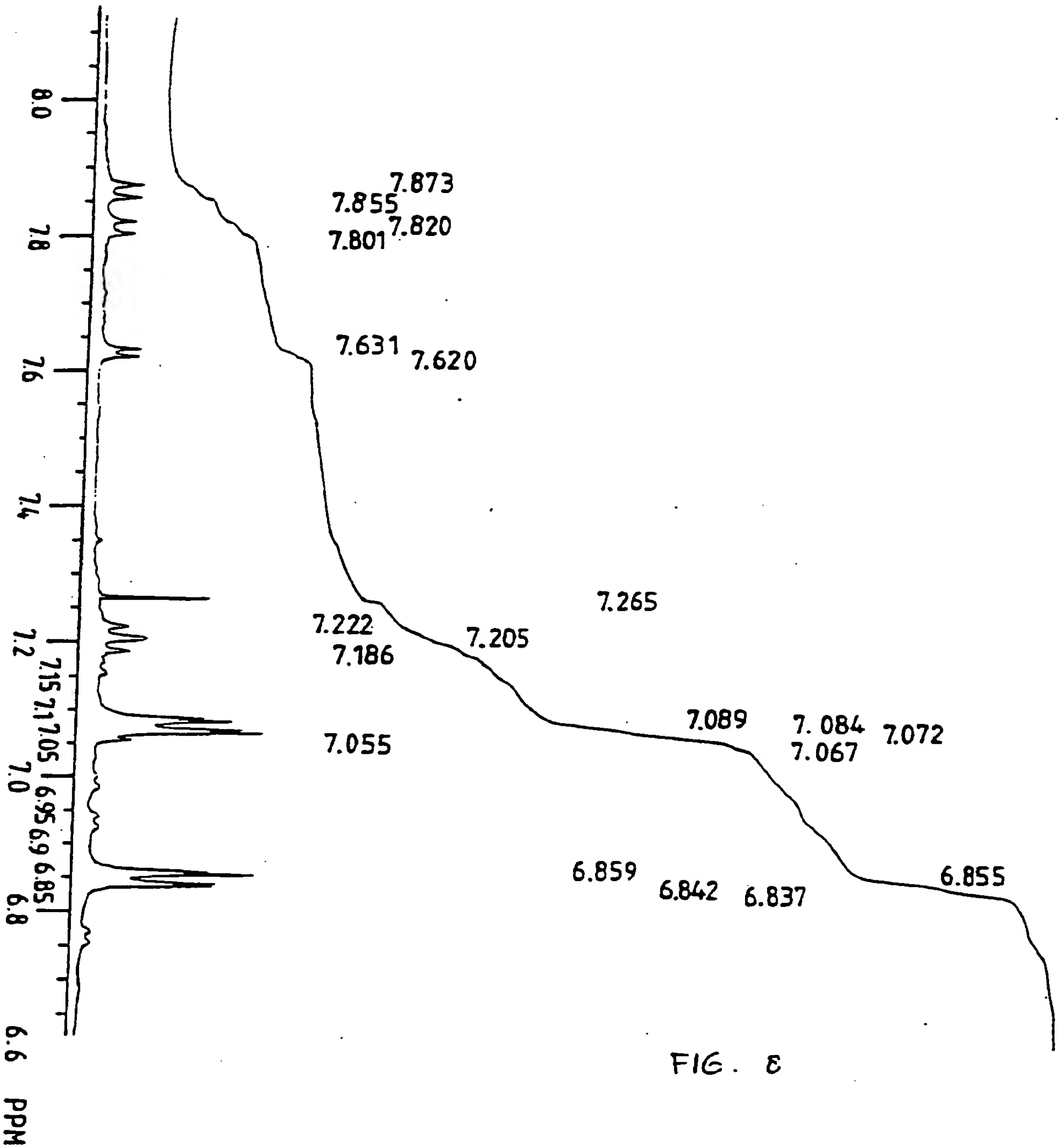

FIG. 8

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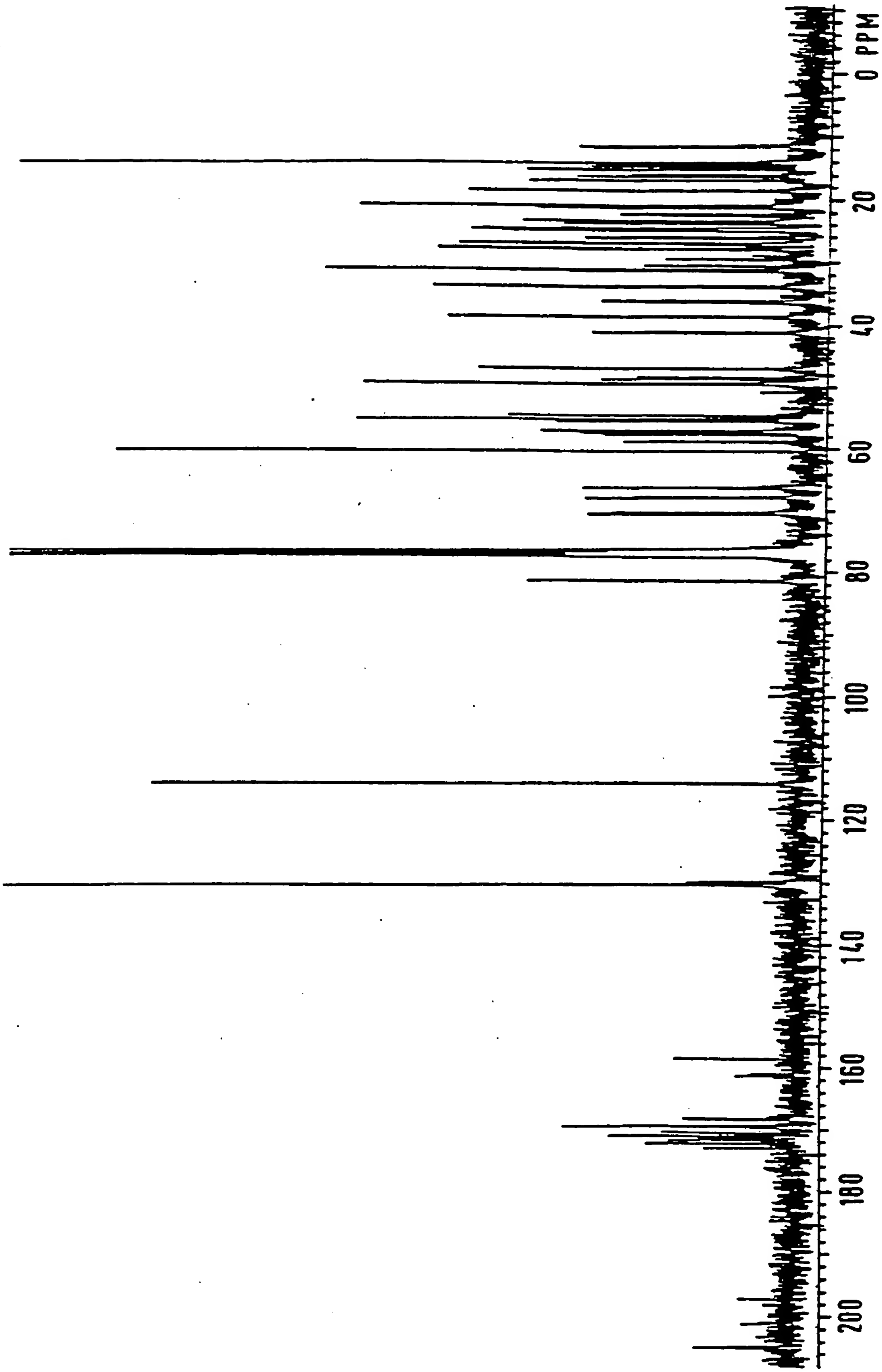

FIG.9

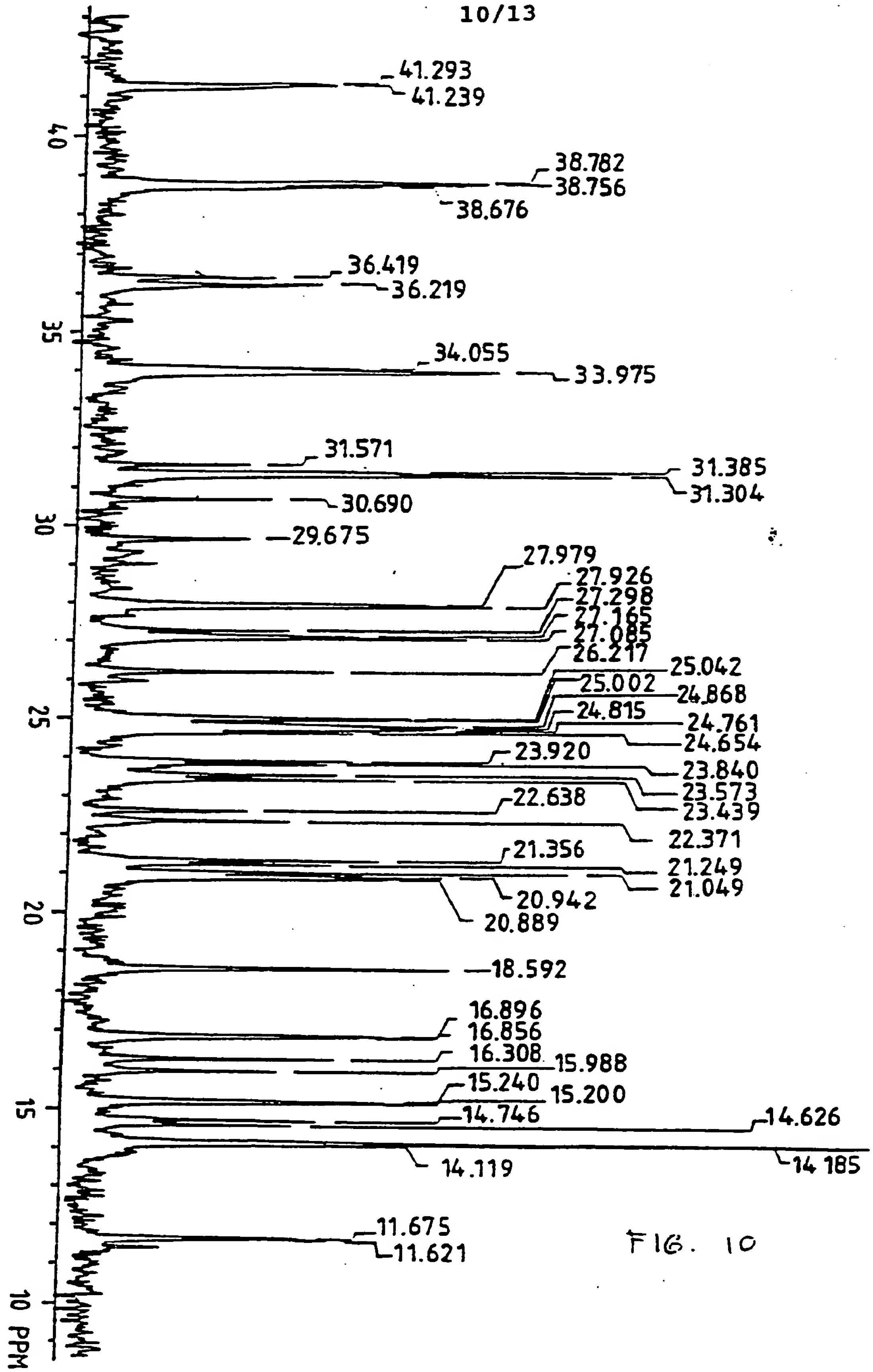

FIG. 10

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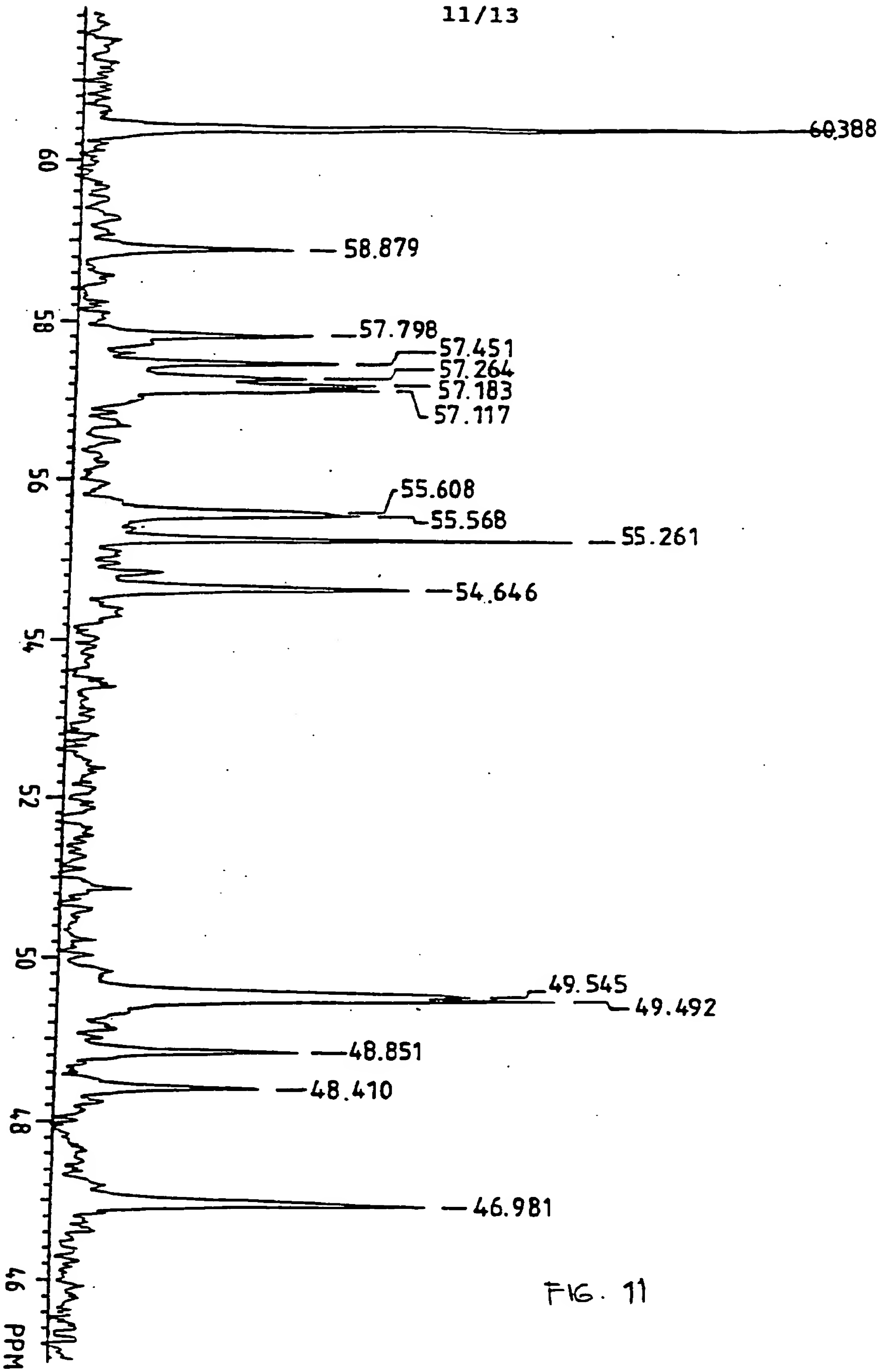

FIG. 11

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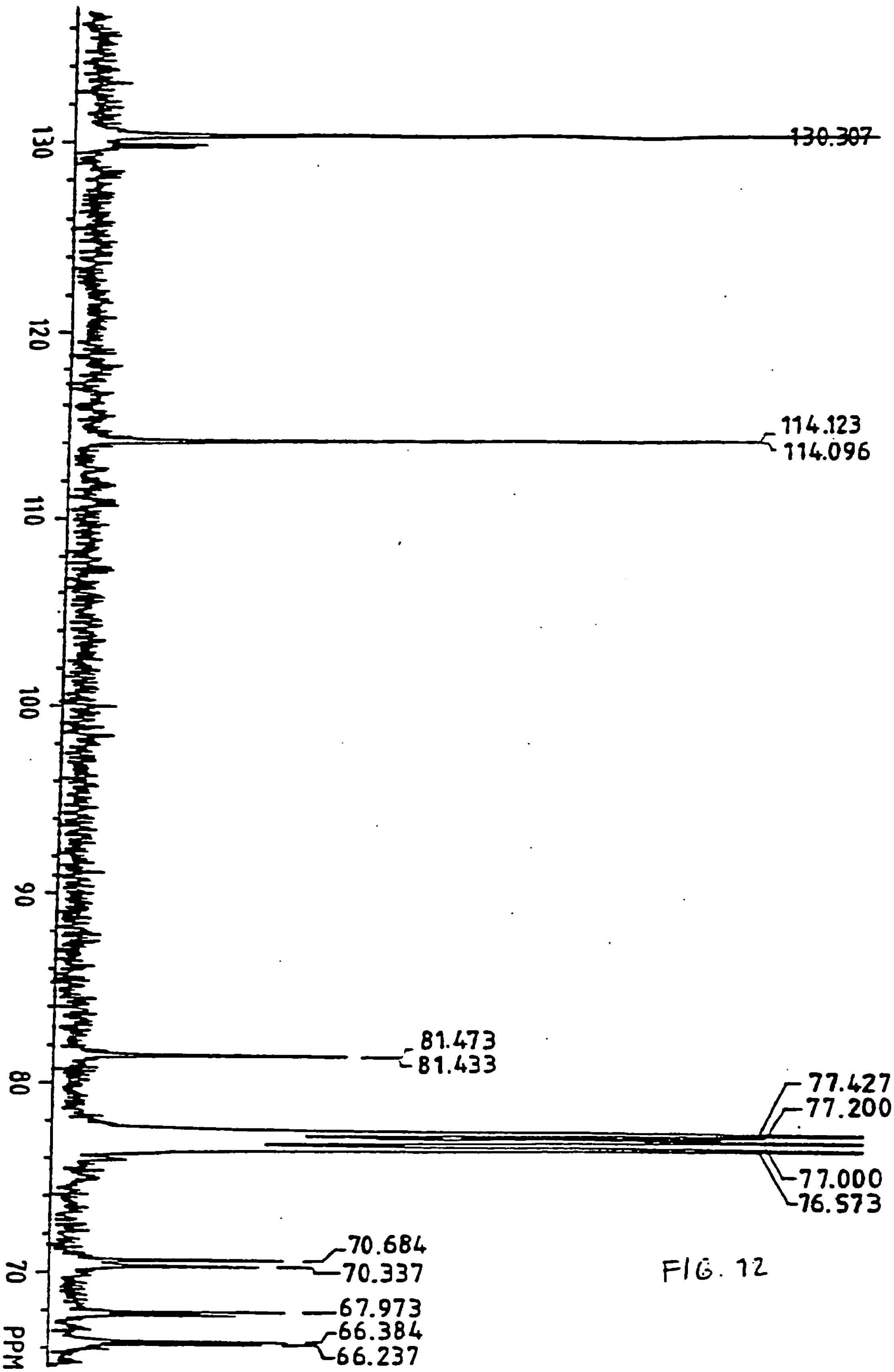

FIG. 12

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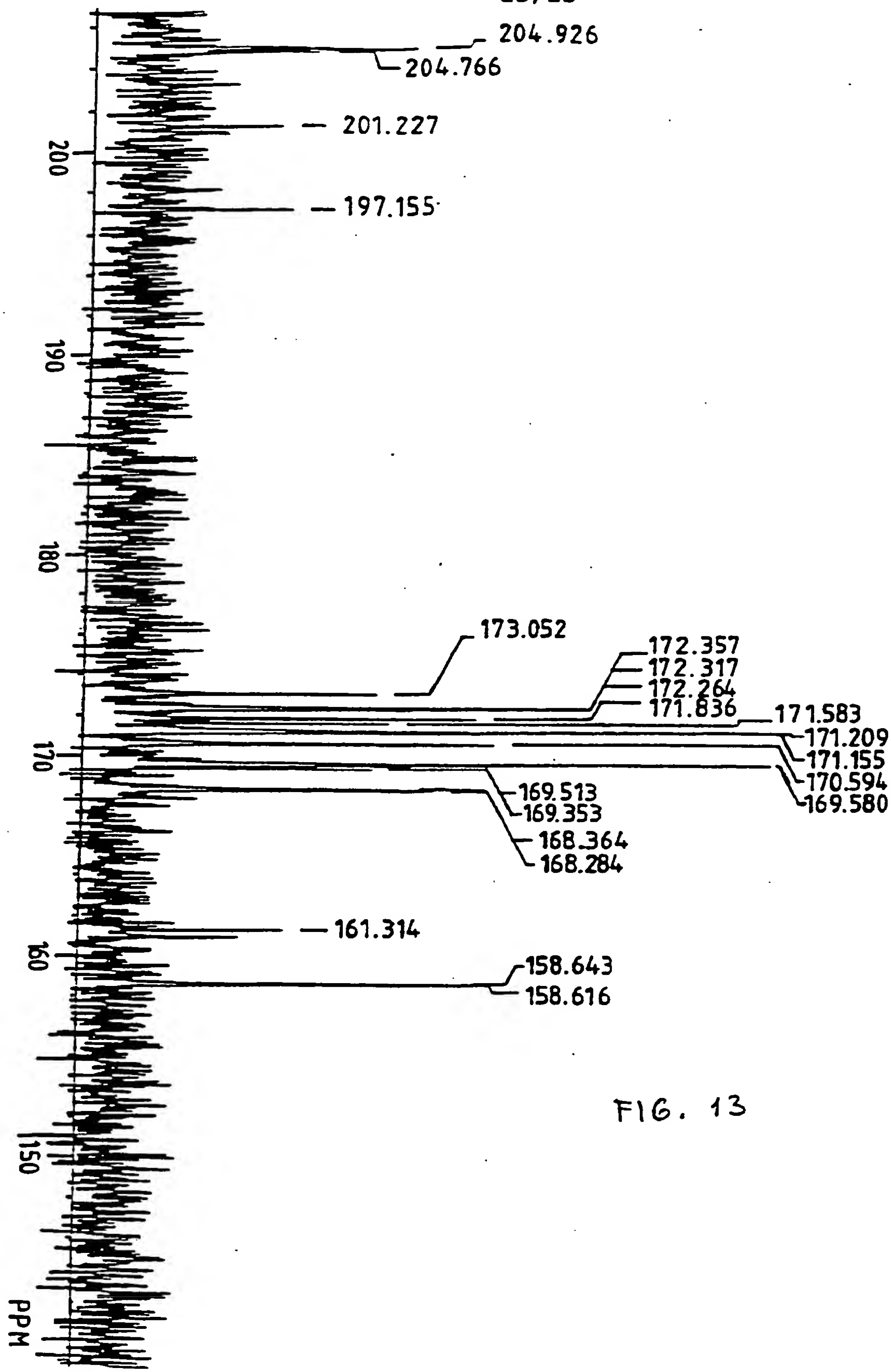
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190
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PPM

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01495

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 07 K 11/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 07 K, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Proceedings of the Tenth American Peptide Symposium, St. Louis, Missouri, 23-28 May 1987, ESCOM, (Leiden, NL), K.L. Rinehart: "Didemnin and its biological properties", pages 626-631 see the whole article -----------	1-5

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15th January 1991	06.02.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	N. TORIBIO Nuria TORIBIO

Form PCT/ISA/210 (second sheet) (January 1985)

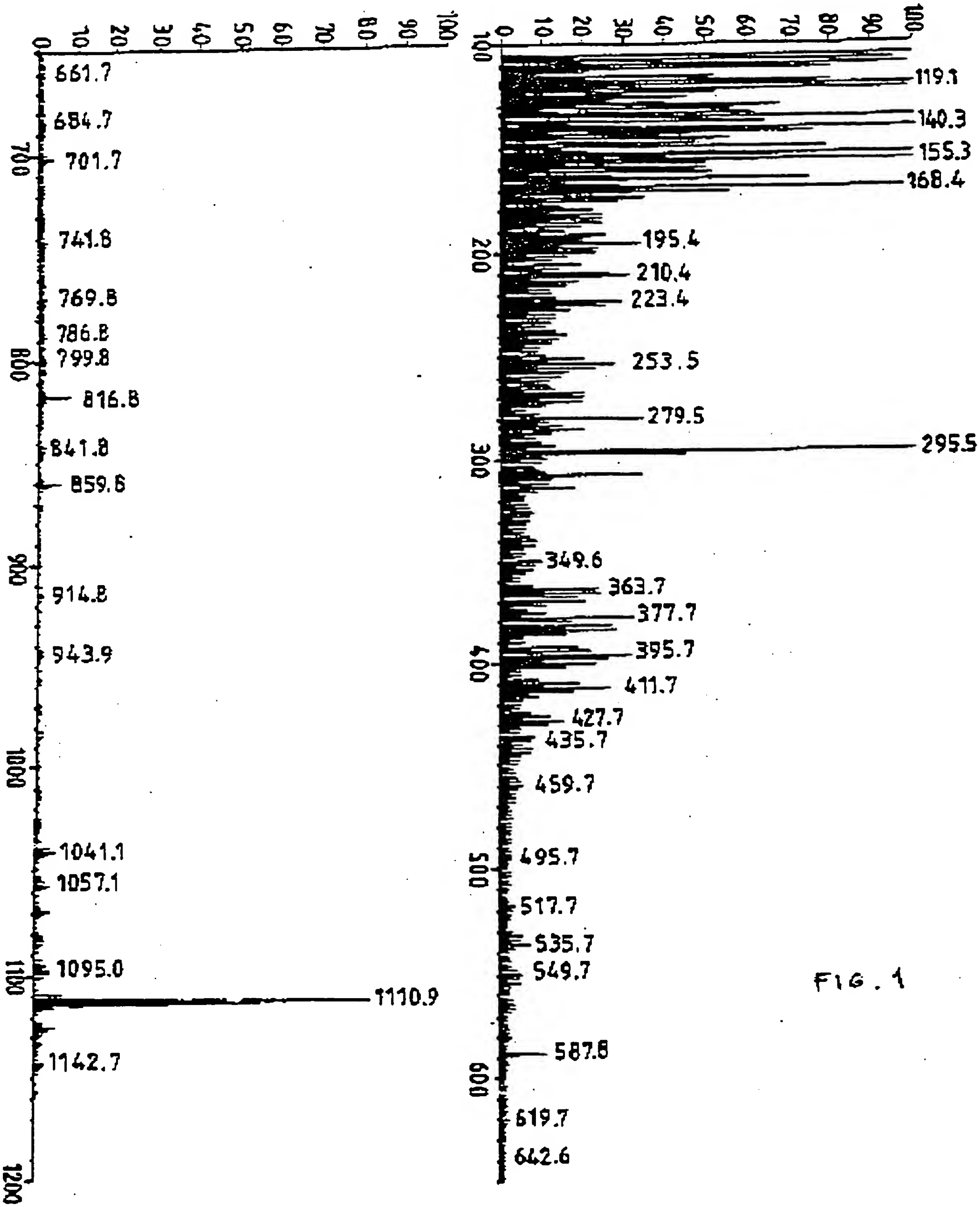
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FIG. 1

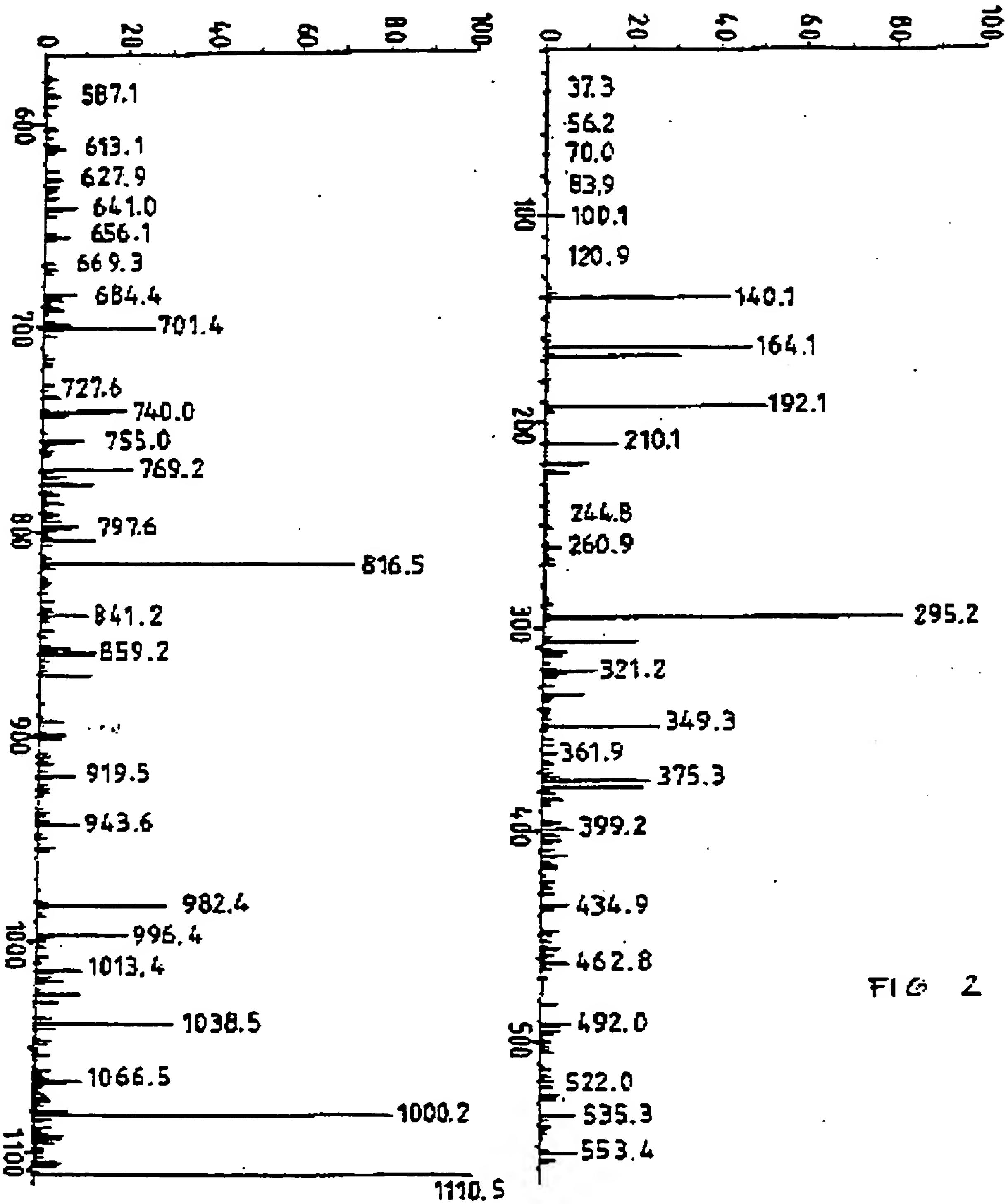
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FIG 2

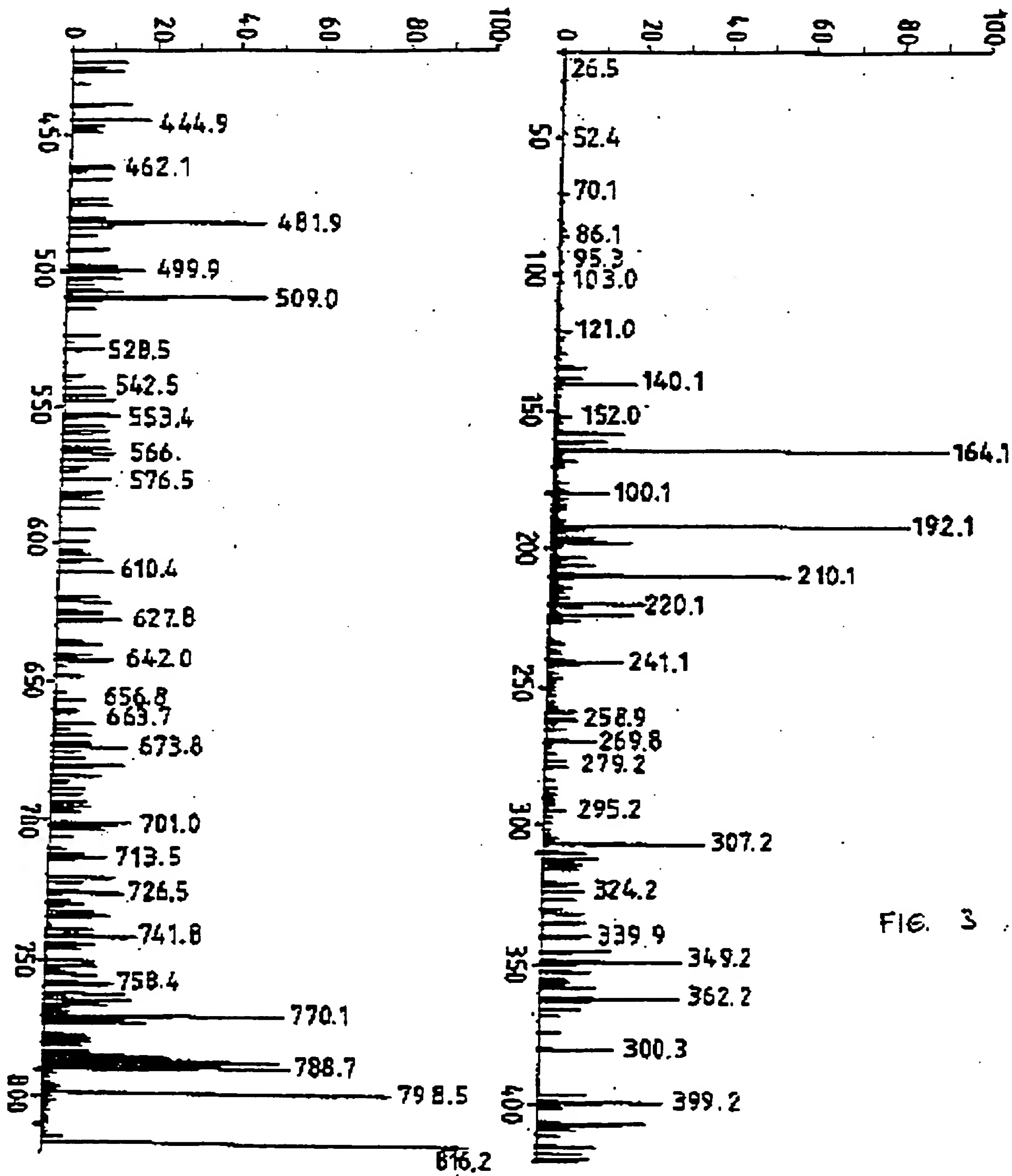

FIG. 3

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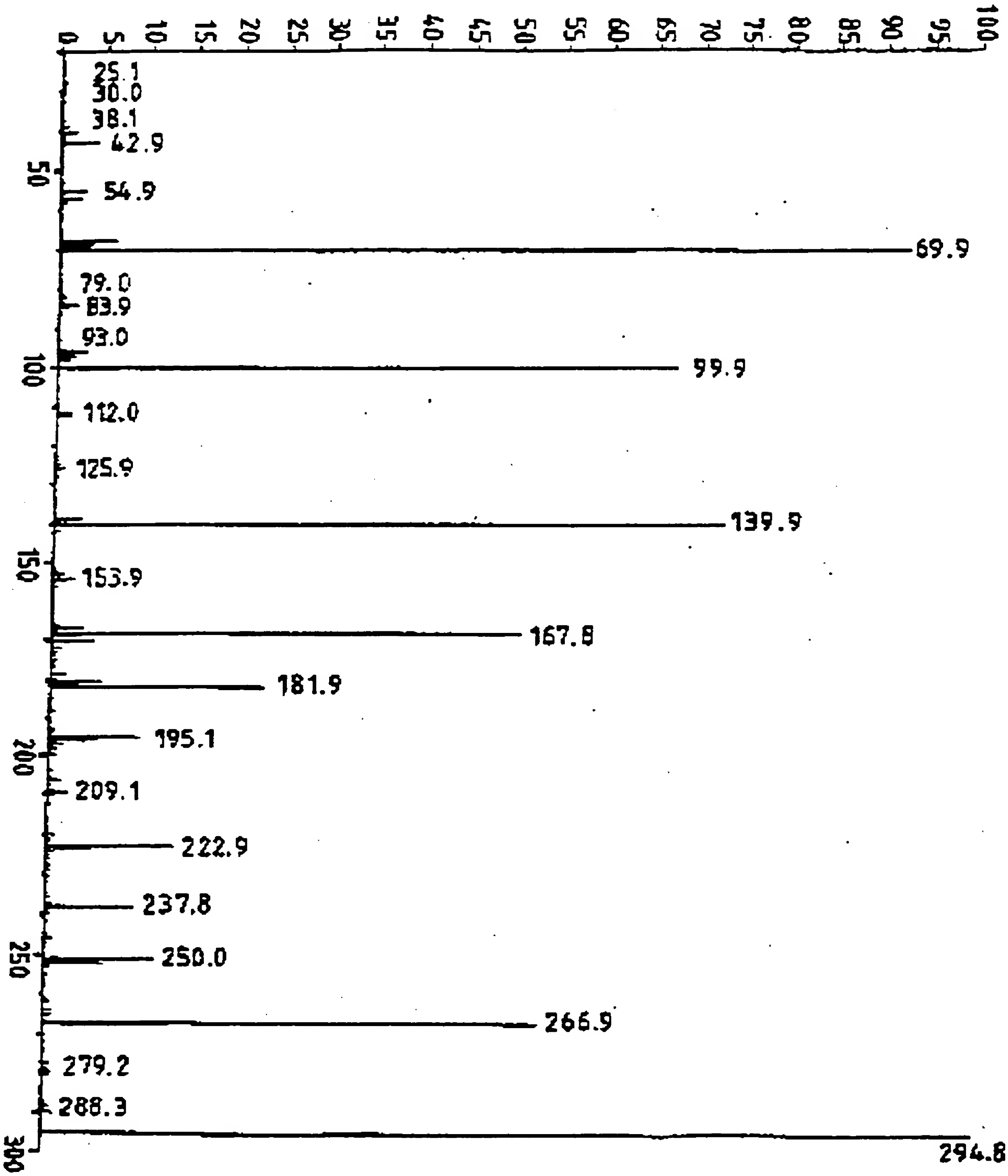

FIG. 4

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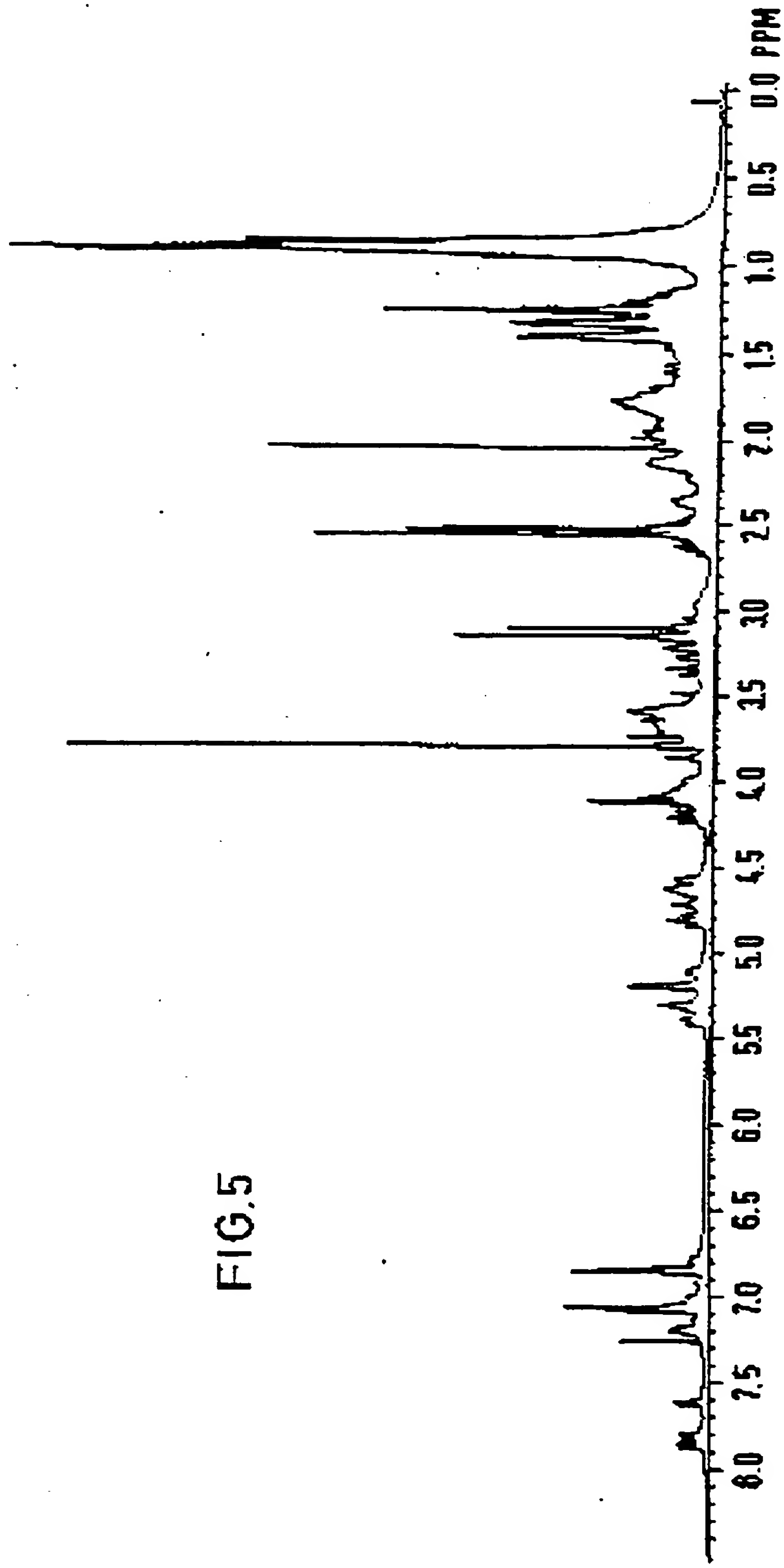


FIG.5

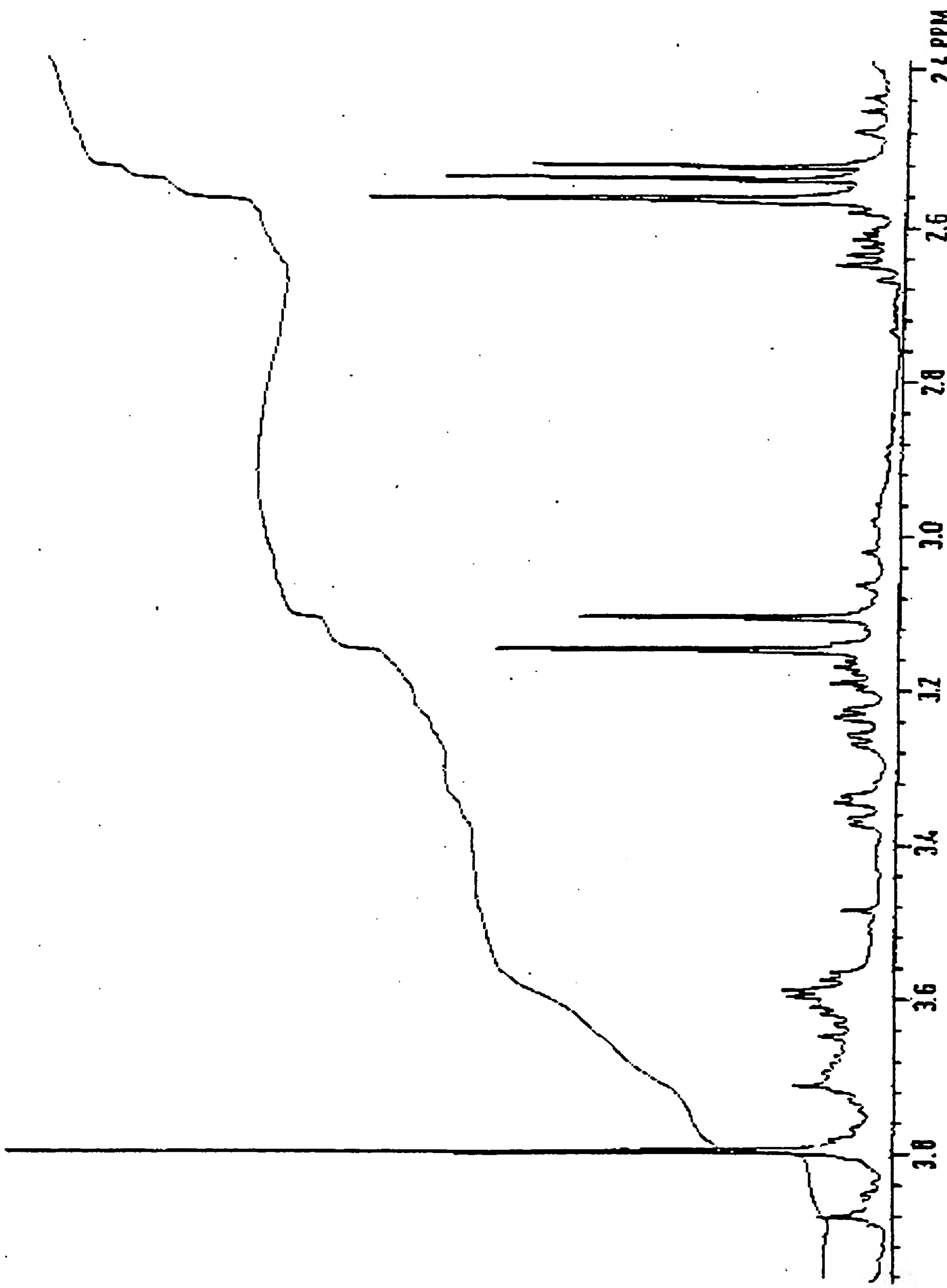


FIG.6

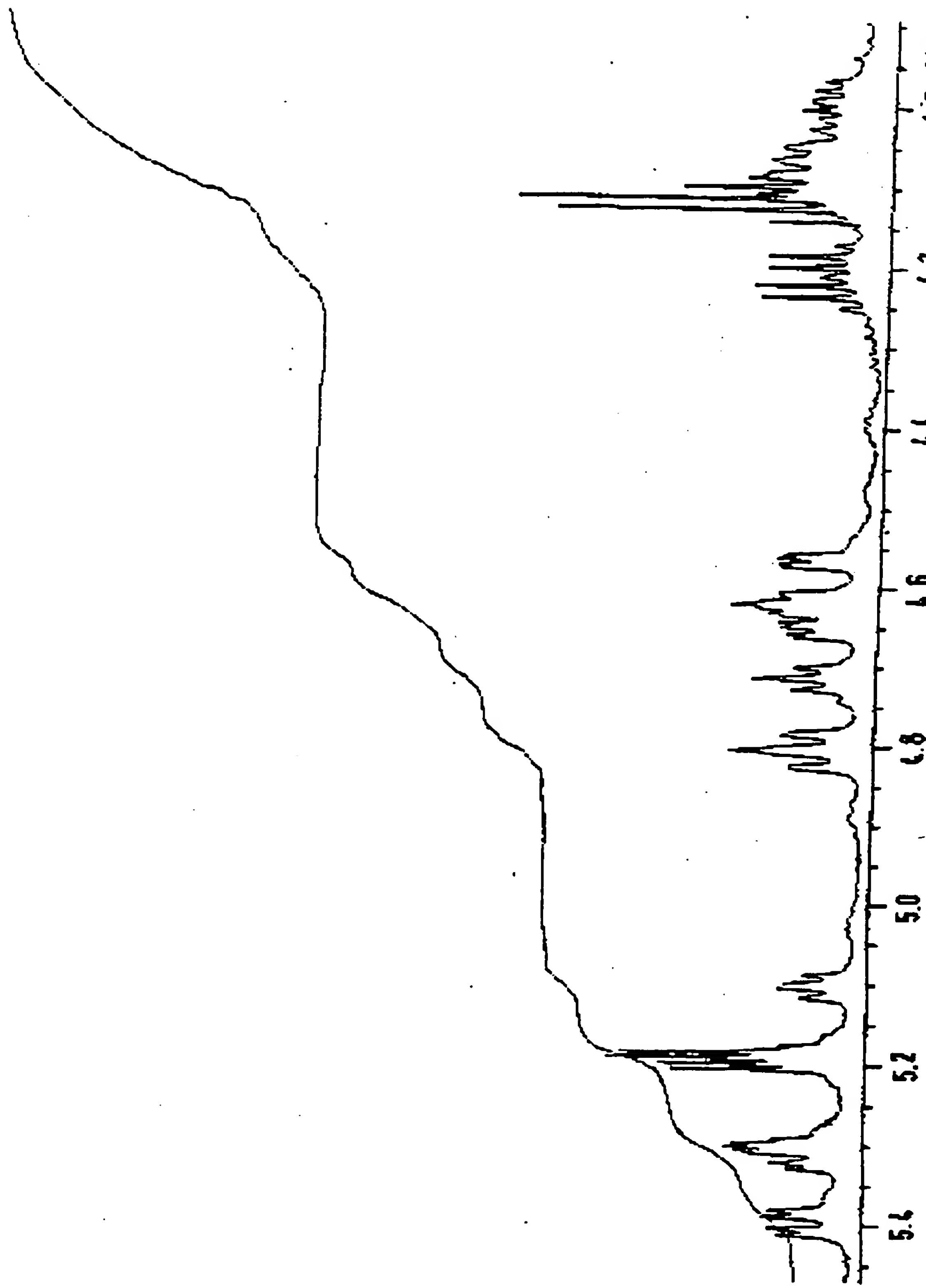
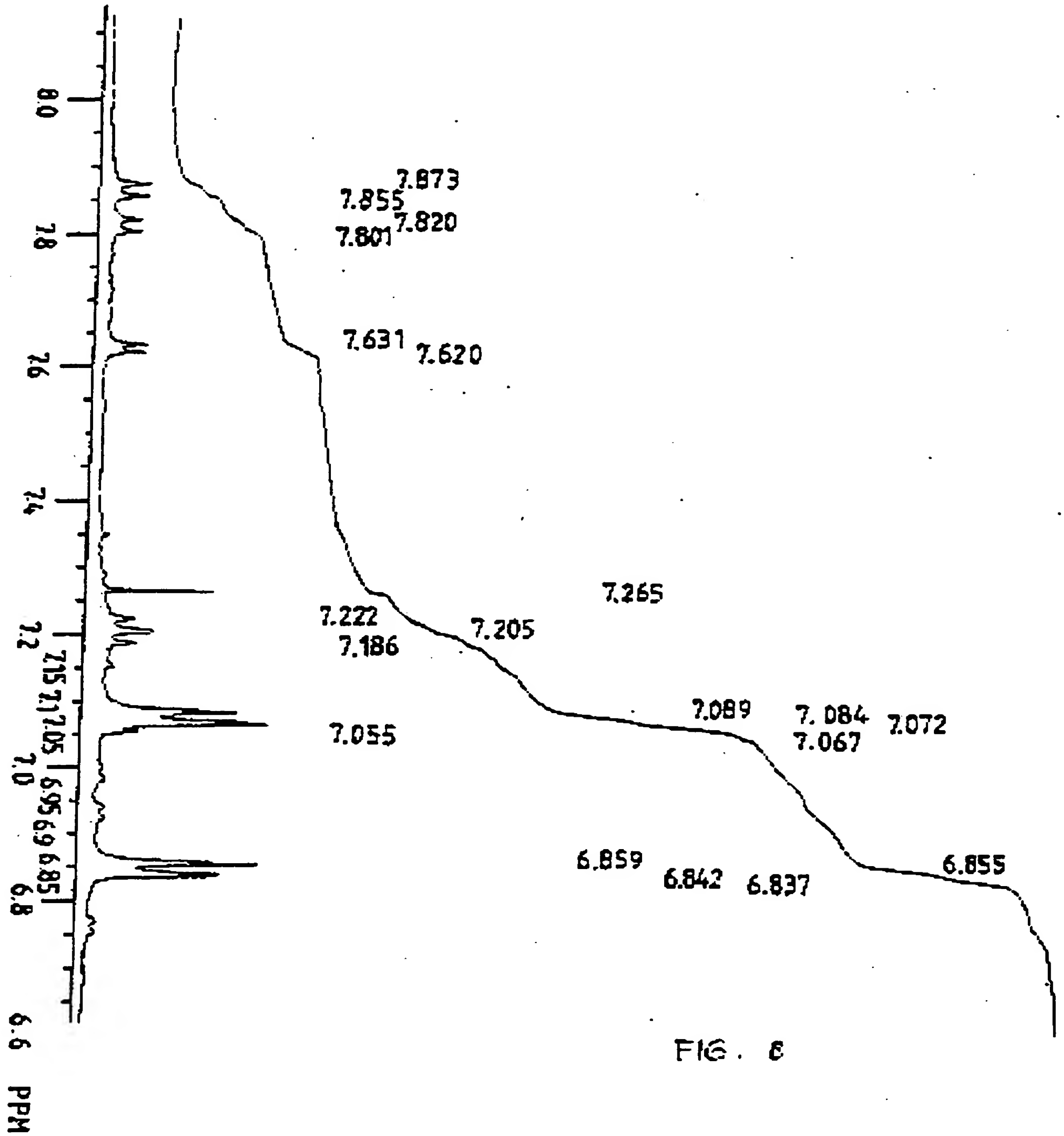


FIG.7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7.072
7.067
6.859
6.842
6.837
6.855
8.0
7.8
7.6
7.4
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6.8
6.6
PPM

FIG. 8

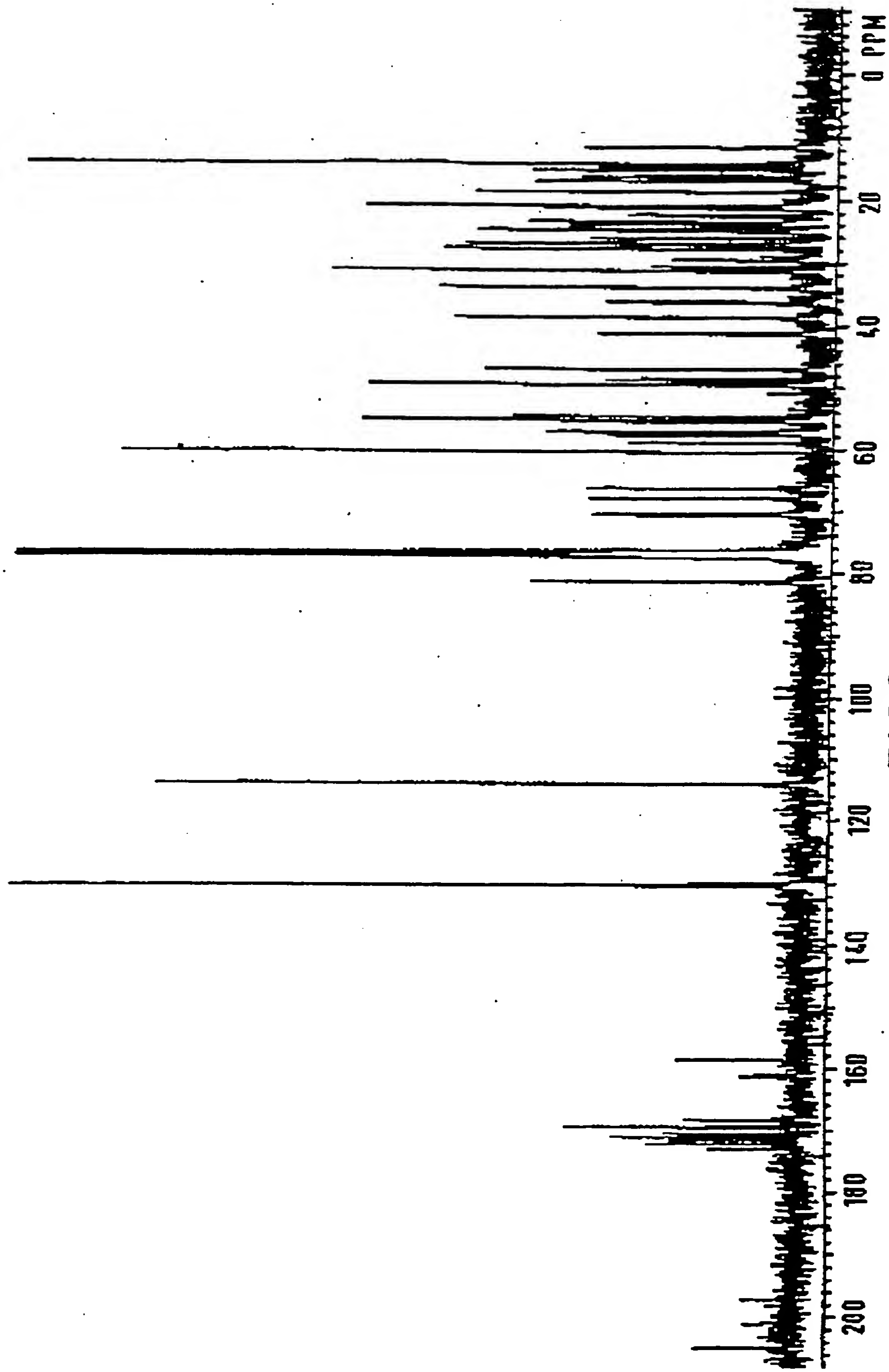

FIG.9

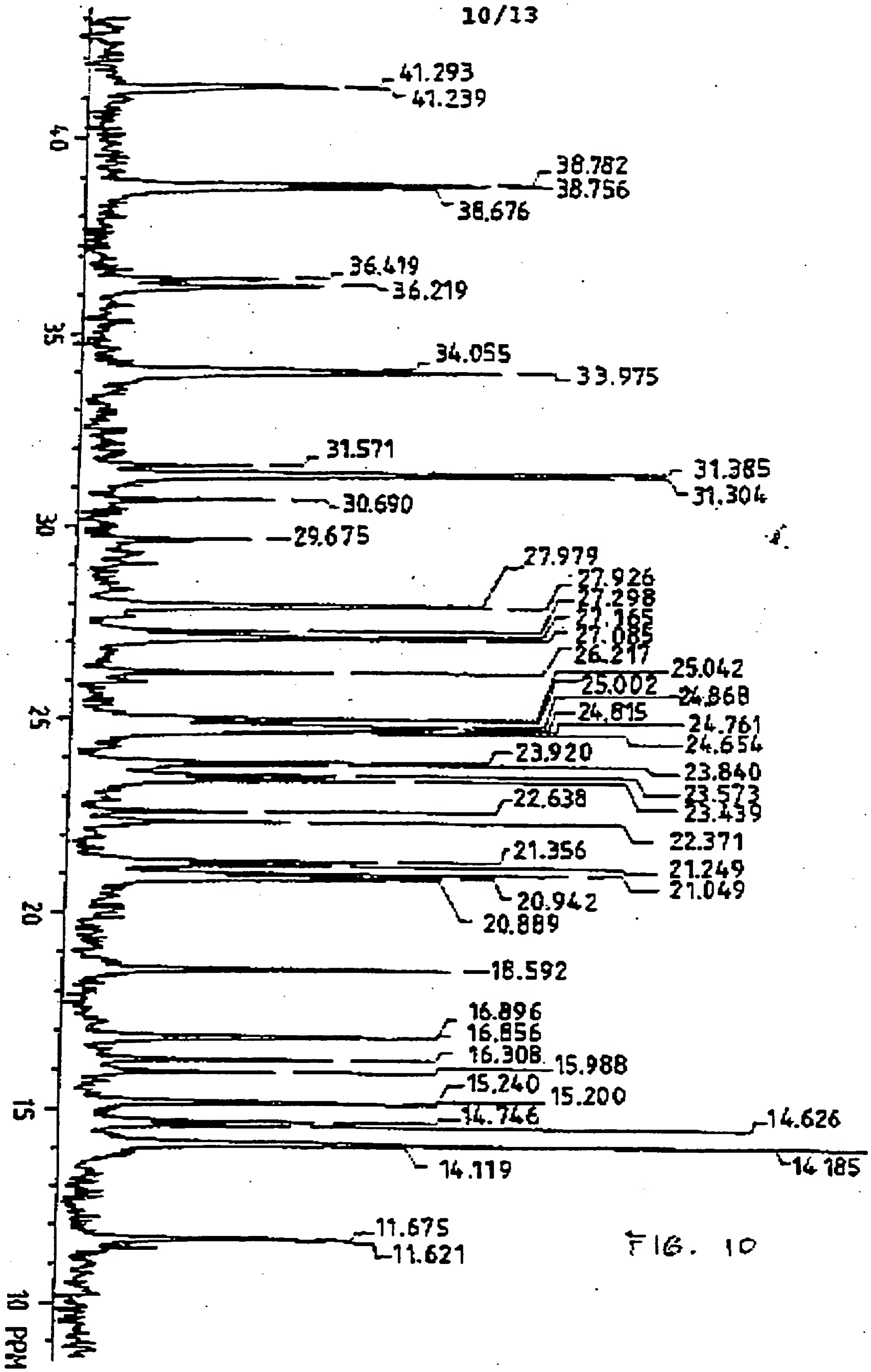

FIG. 10

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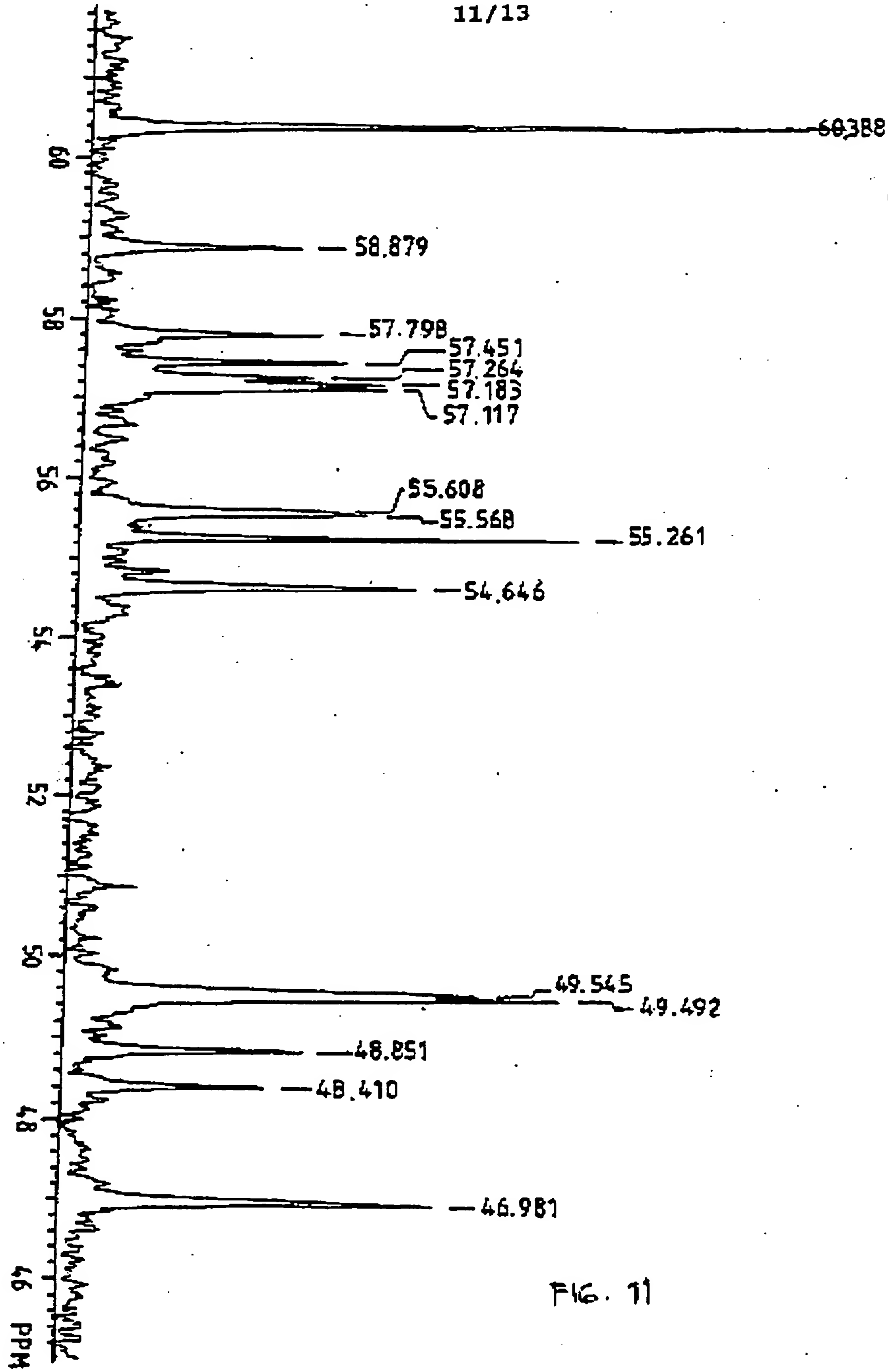

FIG. 11

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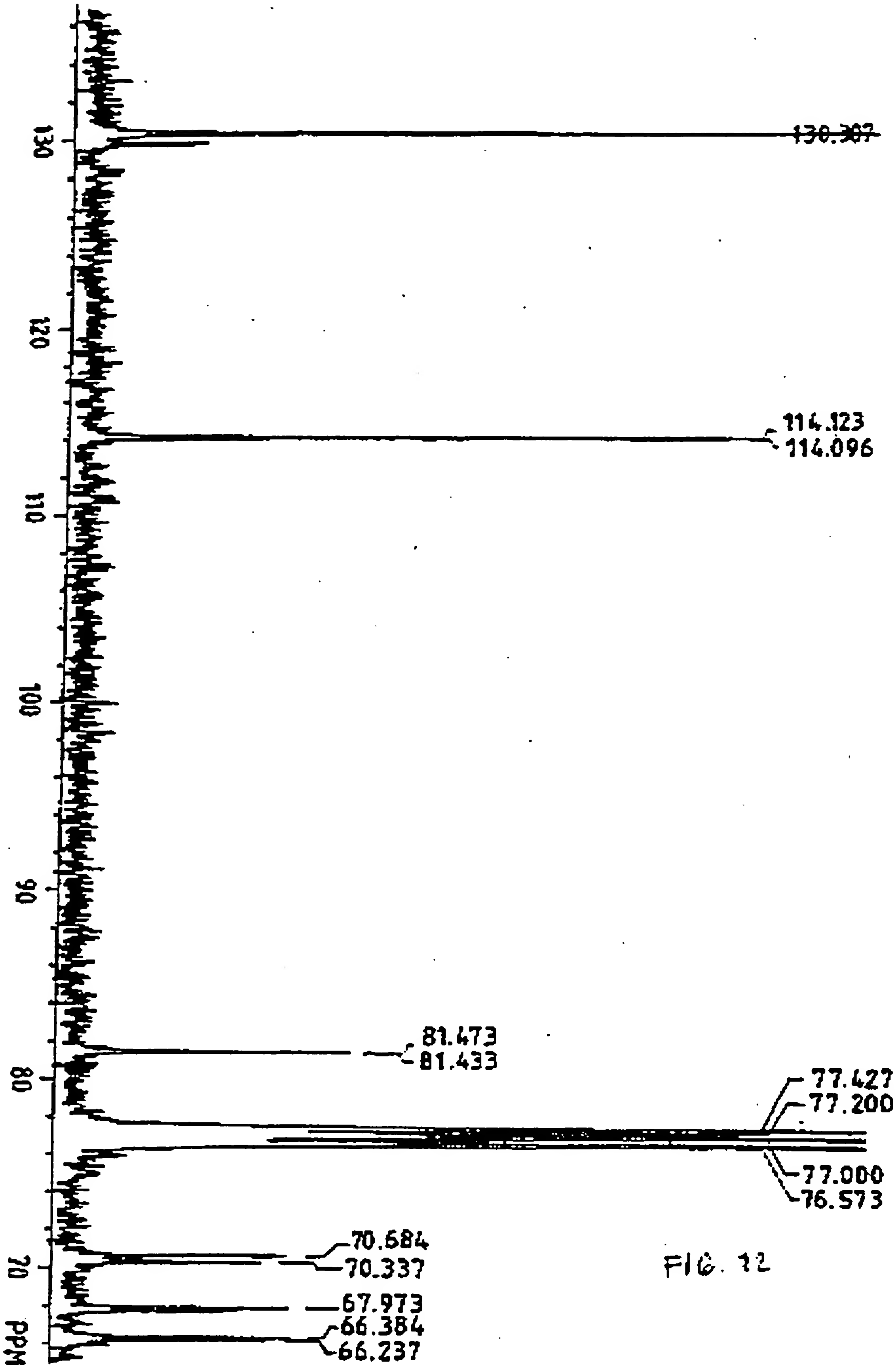
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130
120
110
100
90
80
70
PPM

FIG. 12

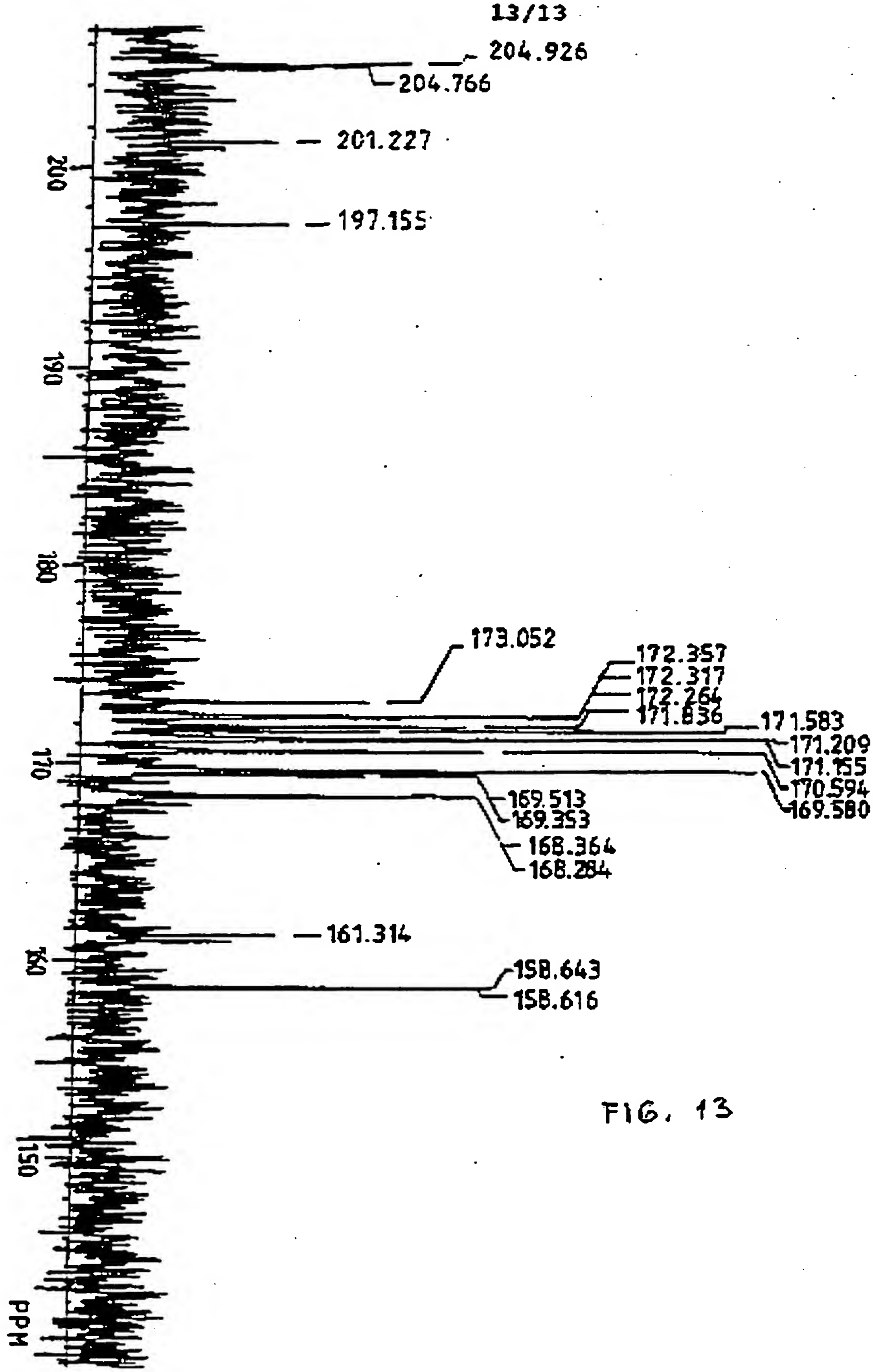

FIG. 13

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